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OXIDATION OF o-PHTHALATE AND RELATED SUBSTANCES BY BAKER'S YEAST

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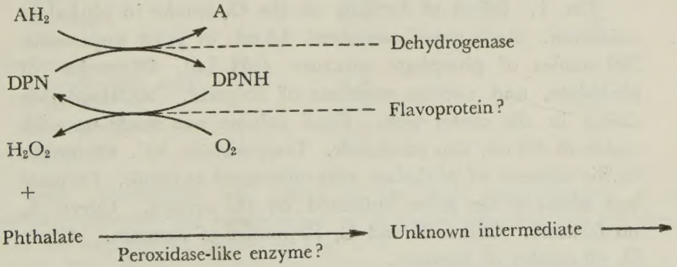
(Received for publication, April 7, 1958)

Evans (*1*) reported recently that *o*-phthalate is metabolized by some soil bacterium, and as the primary oxidation product of the substance he isolated 4,5-dihydroxyphthalate. We have observed that *o*-phthalate is also oxidized by baker's yeast, through a peculiar mechanism which has not been recorded thus far in the literature pertaining the oxidative metabolism of yeast cells. The discovery of this phenomenon was a matter of contingency: on investigating the process of formate oxidation by baker's yeast at different pH's using various kinds of buffers, we found that the rate of O_2-uptake was considerably greater with phthalate buffer than with glycine or citrate buffer. The excess O_2-uptake occurring in phthalate buffer was hardly attributable to the oxidation of the added formate alone and was markedly dependent on the concentration of phthalate applied. It was revealed that phthalate was actually oxidized coupled with the oxidation of formate with participation of DPN and intermediary formation of hydrogen peroxide. Besides formate —which may be called the "co-substrate" of phthalate oxidation—a number of substance, whose oxidation has been known to be DPN-linked, were found to cause the same phenomenon. Based on these observations, and the many others to be described later, it was inferred that the phthalate oxidation baker's yeast probably occurs according to the following mechanism:



Similar oxidation processes invoked by the oxidation of "co-substrate" occurred with 4- and 3-hydroxyphthalates, phenylglycine-*o*-carboxylate and *p*-hydroxybenzoate, but with benzoate, phenylacetate, anthranilate, homogentisate and catechol. Described herein are the experimental data which led us to the conclusion represented by the above schema.

EXPERIMENTS AND RESULTS

A) *Experiments with Intact Cells.*

Baker's yeast (*Saccharomyces cerevisiae*, Hansen) of Oriental Yeast Company was used throughout this study. Cells were washed twice with distilled water, and after being aerated for 12 hours at 25°, they were further washed five times with distilled water and suspended in *M*/50 phosphate buffer* of pH 3.0 in an amount of about 100 mg dry weight of cells per ml. When stored at 0°, the cell suspension thus prepared retained its activity of phthalate oxidation almost unchanged for several days. O_2-uptake was measured at 30° using Warburg's manometer (with air in the gas-space).

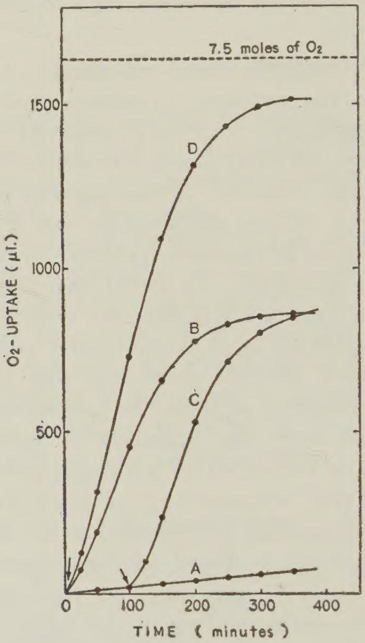


Fig. 1. Effect of formate on the O_2-uptake in phthalate oxidation. Each vessel contained 0.5 ml. of yeast suspension, 200 μmoles of phosphate mixture (pH 3.0), 10 μmoles of phthalate, and various amounts of formate. KOH-solution (10%) in the center well. Final volume was made up with water to 5.0 ml. Gas phase, air. Temperature, 30°. O_2-uptake in the absence of phthalate was subtracted as blank. Formate was added at the point indicated by the arrows. Curve A, no formate. Curves B and C, 10 μmoles of formate. Curve D, 40 μmoles of formate.

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* H_3PO_4-KH_2PO_3 mixture was a weak but suitable buffer for the pH-range 3—4 in our experiments.

I. Enhancement of O_2-uptake Occurring in the Co-existence of Phthalate and Formate—When yeast cells were incubated with phthalate in the presence of oxygen, there occurred only a little O_2-uptake (Curve A, Fig. 1), but the addition of formate caused a remarkable increase in the rate of O_2-uptake, the excess O_2-consumption proceeding with a steady rate for a certain period of time before it gradually tapered off (Curves B and C, Fig. 1)*. The amount of the net O_2-uptake per mole of phthalate increased with the concentration of formate added. The maximum O_2-uptake caused by the addition of excess formate was about 6.7 moles of O_2 per mole of phthalate, and the RQ-value observed was 0.94**. If phthalate had been completely oxidized to CO_2

TABLE I

Effects of Various Substances on the Oxidation of Phthalate

Substance	Relative effect[1]	Relative rates of O_2-uptake[2] in the absence of phthalate
a) Aldehyde		
Formaldehyde	9.8	10
Butyraldehyde	8.0	16
Crotonaldehyde	5.7	8
Benzaldehyde	7.0	15
Vanillin	18.0	15
b) Alcohols		
Ethanol	8.3	>30
Butanol	7.1	16
Propanol	4.0	13
Methanol	0.0	0
c) Sugars		
Glucose	12.2	50
Fructose	13.0	>50
Sucrose	15.7	>60
d) Acids		
Acetic	10.0	30
Lactic	6.3	12
Pyruvic	13.6	9
Glycolic	1.3	5
Glyoxylic	0.0	6
Malic	0.0	0
Succinic	0.0	0
Citric	0.0	0
e) Reducing agents		
Thioglycolic acid	4.0	>1
Cysteine	2.0	1~2
Na-thiosulfate	3.1	0
Ascorbate	2.8	2~3

Each co-substrate was added in the final concentration of 10 μmoles; otherwise the composition of reaction mixtures was the same as in the experiment shown in Fig. 1.

1) The effect caused by the addition of formate was taken as 10.

2) The rate of O_2-uptake caused by the addition of formate in the absence of phthalate was taken as 10.

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* In the presence of formate alone, about 0.5 moles of O_2 was consumed per mole of formate.

** The relation between the amount of O_2-uptake and the amount of decreased phthalate was not yet established.

and water, 7.5 moles of O_2 must have been consumed per mole of phthalate and the RQ-value must have been 1.07.

The enhanced O_2-uptake occurred most markedly in the pH-range between 2.7–3.1, and ceased almost completely at pH's above 5.5. The fact that the optimal pH was rather low is probably due to the increased penetration of free phthalic acid into yeast cells in those pH-regions.

II. Various Substances Acting as "Co-substrate" of Phthalate Oxidation—The enhanced O_2-uptake as it was observed in the above experiment was also brought about by a number of substances besides formate. In Table I are compared the effects of various substances on phthalate oxidation, the effect of formate (10 μ moles) being taken as 10. The relative rates of O_2-uptake caused by each substance in the absence of phthalate are also listed in the last column of the table. It is apparent from these data that with only a few exceptions those substances which caused enhanced O_2-uptake in the presence of phthalate are by themselves good substrates for the respiration of yeast cells. Noteworthy is the fact that all these substances are known to require pyridine nucleotide for their direct dehydrogenation or for some reaction step involved in the process of their oxidative breakdown. It should also be noticed that the effect of various reducing agents such as thioglycolate, cysteine, thiosulfate and ascorbate, which caused only a slight increase of O_2-uptake in the absence of phthalate.

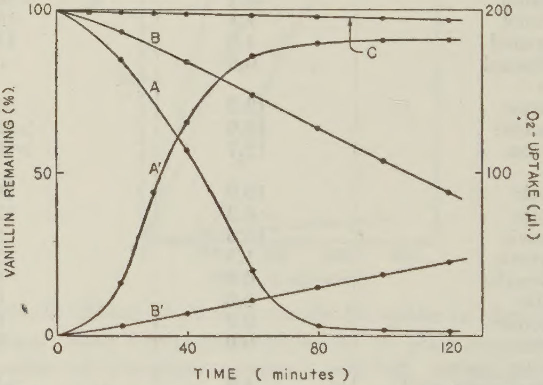

FIG. 2. Disappearance of vanillin under aerobic and anaerobic condition. 20 μmoles of phthalate and 5 μmoles of vanillin were added. Other composition of reaction mixture was the same as in the experiment shown in Fig. 1. The amount of remaining vanillin was measured colorimetrically by the method of Friedemann and Haugen (*3*) after centrifuging off the yeast cells. Curve A, with phthalate under aerobic condition. Curve B, without phthalate under aerobic condition. Curve C, with phthalate under anaerobic condition. Curves A′ and B′, O_2-consumption with and without phthalate, respectively.

Using vanillin as an effective co-substrate, its fate—in the presence or phthalate—under aerobic and anaerobic conditions was followed, by measuring its quantity colorimetrically using the method of Friedemann and Haugen (*3*). The results obtained are illustrated in Fig. 2, from which it may be seen (i) that the consumption of vanillin occurred only in aerobic, not in anaerobic, condition, and that (ii) under aerobic condition both the consumption of vanillin and the O_2-uptake were markedly accelerated by the presence of phthalate.

These observations suggest that the factor causing the oxidation of phthalate may be some substance occuring in the intermediate step of oxidation of co-substrate; and such a factor may be related to a reduced coenzyme of DPNH or TPNH, since (i) the substance acting as the co-substrates are all those requiring DPN or TPN in their process of oxidation (dehydrogenation), and (ii) the reducing substances such as thiosulfate which are known to be capable of reducing DPN or TPN were also found to cause the enhanced O_2-uptake in the presence of phthalate.

III. Evidence for the Process of Phthalate Oxidation—In the above experiments it was shown that the oxidation of phthalate is provoked either by the addition of "co-substrate" or by the presence of reducing agent such as thiosulfate. The experimental data presented in Table II show that a similar

TABLE II

Replacement of Co-substrate by Hydrogen Peroxide

(Net O_2-uptake in μl.)

Time in Min.	$+H_2O_2$	$+H_2O_2+$Azide
10	12.7	4.7
20	27.7	8.5
30	38.9	13.0

Concentrations of phthalate and buffer were the same as in the experiments shown in Fig. 1. Both hydrogen peroxide and azide were added in final concentrations of 5 and 1 μmoles, respectively. Positive O_2-output caused by the decomposition of H_2O_2 by catalase in the absence of phthalate was subtracted as blank.

TABLE II′

Effect of Methanol on the Phthalate Oxidation in the Presence of Co-substrate

(Net O_2-uptake in μl.)

Time in Min.	+Formate	+Formate+Methanol
10	43.1	17.0
20	121.0	70.8
30	180.9	105.7

Concentrations of phthalate, buffer and formate were the same as in the experiments shown in Fig. 1. Methanol was added in the final concentration of 10 μmoles.

effect is also brought about by the effect of hydrogen peroxide in the absence of co-substrate. The provoking effect of hydrogen peroxide was found to be markedly suppressed by azide, indicating that some metallo-enzyme is participating in the process. These results give us ground for the supposition that the agent causing directly the oxidation of phthalate may probably be hydrogen peroxide, and that in the previous experiments it might have been formed as a product of DPNH or TPNH by molecular oxygen. The intermediate formation of hydrogen peroxide in the oxidation of co-substrate and the effect of hydrogen peroxide of provoking phthalate oxidation were made probable by the observation that the O_2-uptake in simultaneous existence of phthalate and formate was suppressed by methanol, a substance which is known to cause, by being acted upon by catalase, the trapping of hydrogen peroxide (Table II′). (It has been ascertained that methanol ($M/500$–$M/1000$) considerably suppressed the process of O_2-liberation from H_2O_2 by catalase in yeast cells.)

IV. Inhibitors of Phthalate Oxidation—Effects of various substances upon the process of phthalate oxidation occurring in the presence of formate were investigated, and the results obtained are summarized in Table III. As may be seen, the process was strongly inhibited by cyanide, azide, fluoride, monoiodoacetate, Hg^{++}-ion and various aromatic monocarboxylic acides, while only a slight or no inhibition was brought about by α,α'-dipyridyl, versene, or diethyldithiocarbamate. It should be added that the aromatic monocarboxylic acids were not competitive with phthalate in their inhibitory action. General discussions on the action of inhibitors will be made in a later section.

TABLE III

Effects of Various Inhibitors

Inhibitors	Final concentration	Phthalate concentration	% inhibition
a) Cyanide	$M/2500$	$M/1000$	100
Azide	,,	,,	,,
Fluoride	,,	,,	98
b) α, α'-dipyridyl	$M/500$	,,	16
Versene	,,	,,	5
Diethyldithiocarbamate	,,	,,	0
c) Monoiodoacetate	$M/5000$	,,	100
Hg^{++}-ion	,,	,,	,,
d) Salicylate	$M/2000$	,,	100
Benzoate	,,	,,	,,
,,	$M/5000$	,,	85
,,	,,	$M/100$	78
Phenylacetate	$M/2000$	$M/1000$	80

Composition of reaction mixtures was the same as in the experiments shown in Fig. 1.

V. Oxidizability of Some Compounds Related to Phthalate—Some aromatic carboxylic acids which were thought to be more or less closely related to phthalate were tested for their oxidizability in lieu of phthalate. It was found that 4- and 3-hydroxyphthalates*, **, phenylglycine-*o*-carboxylate and *p*-hydroxybenzoate were oxidized, like phthalate, requiring the presence of co-substrate and showing the optimum pH at around 2.8. The rates of oxidation of these compounds were, however, 25 per cent, 10 per cent, 15 per cent and 28 per cent, respectively, of that of phthalate. 4- and 3-hydroxyphthalates were also oxidized by hydrogen peroxide in the presence of yeast but at a little lower rate of O_2-uptake than in the case of phthalate oxidation. Among these aromatic compounds related to phthalate, 4- and 3-hydroxyphthalates are surmised to be possible intermediates occurring in the oxidation of phthalate. Slight oxidation was observed with salicylate (at pH 5.0), but the process was essentially different from that of the above-mentioned substances in that it occurred in the absence of co-substrate. Moreover, salicylate exerted strong inhibition upon the process of phthalate oxidation. Other aromatic compounds such as benzoate, phenylacetate, anthranilate, homogentisate and catechol were found to be entirely inert towards yeast cells, regardless of the pH of the medium and of the presence or absence of co-substrate.

B) Experiment with Cell-freeEnzyme System

To get further insight into the mechanism of phthalate oxidation, attempt was made to separate the enzyme system from yeast cells and perform *in vitro* experiments under simpler conditions. Thus far the enzyme system could be separated into three fractions, Fractions I and II and coenzyme DPN. Fractions I and II were obtained by the following procedure.

VI. Preparation of Cell-free Enzyme System—Five grams of packed yeast cells were washed twice with $M/2$ K_2HPO_4 solution, and the cell paste was ground with one or two weights of alumina*** in the cold for 5~7 minutes**** using a chilled mortar and pestle. To the mixture was added 10 ml. of $M/50$ K_2HPO_4, and the pH was adjusted to 7.5 by adding 5 per cent NaOH-solution. All the subsequent steps were carried out at 0°~4°. Alu-

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*, ** 4-hydroxyphthalic acid was prepared by the following procedure: phthalimide $\xrightarrow{\text{nitration } (4)}$ 4-nitrophthalimide $\xrightarrow{\text{reduction } (5)}$ 4-aminophthalimide $\xrightarrow[\text{b) decomposition with water } (5)]{\text{a) diazotization } (5)}$ 4-hydroxyphthalic acid $\xrightarrow{\text{vacuum sublimation } (6)}$ 4-hydroxyphthalic anhydride.

Preparation of 3-hydroxyphthalic acid was performed as follows: phthalic acid $\xrightarrow{\text{nitration } (7)}$ 3-nitrophthalic acid $\xrightarrow{\text{reduction } (5)}$ 3-aminophthalic acid $\xrightarrow[\text{b) decomposition with water } (5)]{\text{a) deazotization } (5)}$ 3-hydroxyphthalic acid $\xrightarrow{\text{vacuum sublimation } (6)}$ 3-hydroxyphthalic anhydride. 4- and 3-hydroxyphthalates were hydrolyzed before use.

*** Aluminium oxide No. 800 of Wako Pure Chemical Industries, LTD.

**** Further grinding led to a marked decrease of enzyme activity.

mina and cells which were not disintegrated by grinding were removed by centrifugation at 500×g for five minutes. The supernate, with its pH maintained at 7.0∼7.5, was stirred for 30 minutes and then subjected to refrigerated centrifugation for 15 minutes at 15,000×g. The precipitate was suspended in M/50 phosphate buffer of pH 7.1, stirred for 30 minutes and centrifuged for 15 minutes at 15,000×g. After repeating the same treatment, the precipitate was resuspended in M/50 phosphate buffer (pH 7.1) and used for experiment as Fraction I. The supernate obtained by refrigerated centrifugation was collected and after being dialyzed against M/50 phosphate buffer (pH 7.1) for 24 hours at 2°, it was used as Fraction II. While Fraction II was relatively stable and could be kept for a week or more at 0° without detectable loss in activity, Fraction I was extremely unstable, losing more than 2/3 of its activity in 24 hours (at 0°) even in a hypertonic sucrose-phosphate mixture (*16*). Because of its instability, the attempt to date to separate Fraction I further into its component have been unsuccessful. Fraction II is a mixture of various enzyme systems. Thus far the following enzymes were found in this fraction: DPN-linked alcohol dehydrogenase, DPN-linked aldehyde dehydrogenase, DPN-linked formic dehydrogenase, TPN-linked aldehyde dehydrogenase, catalase and peroxidase (cytochrome c peroxidase?).

VII. Phthalate Oxidation Catalyzed by the Cell-free Enzyme System—Fraction I or II alone, or the mixture thereof showed only a little O_2-uptake when incubated with phthalate and co-substrate (ethanol or formate), while the addition of DPN to the mixture of Fractions I and II caused enhanced O_2-uptake under otherwise the same condition (See Table IV). It should be remarked that the reaction occurring in this case differed from the corresponding process caused by intact yeast cells in the following points:

(i) The optimal pH for the *in vitro*-oxidation was found to be 7.1, while that of *in vivo*-oxidation was 2.7–3.1.

(ii) In the *in vitro*-oxidation, less than 1 mole of O_2 was absorbed per mole of phthalate and no liberation of CO_2 did occur, while in the *in vitro*-oxidation as much as 6.7 moles of O_2 were consumed and 6.3 moles of CO_2 were evolved per mole of phthalate consumed.

(iii) The enzyme systems, especially those contained in Fraction I, are quite unstable, so that the *in vivo*-experiments, unlike the *in vitro*-experiments, had to be run for only a short period of time.

For the reason mentioned under (iii), all *in vitro*-experiments were carried out at a lower temperature (20°) and with the addition of cysteine and nicotinamide which were found to be effective in protecting, to some extent, the enzyme systems from inactivation.

A boiled extract of the crude preparation of Fraction II, which had not been subjected to dialysis (against M/50 phosphate buffer for 24 hours at 2°) caused a marked enhancement of O_2-uptake when brought together with Fractions I and II and co-substrate. The data demonstrating these facts are summarized in Table IV. It may be remarked that the enhancement of O_2-uptake caused by the boiled extract was appreciably greater than that brought about by DPN, and further addition of TPN or metal ions such as Fe^{++},

Cu^{++}, Zn^{++}, Mg^{++}, Mn^{++}, Co^{++} and Fe^{+++} could not improved the effect of DPN. This fact suggests that the boiled extract of the crude preparation of Fraction II contained some unknown factor other than DPN or TPN. By treatment of the boiled extract with ion exchange resins (Amberlites IRA-410 and IR-120) it was found that all the effective cofactors contained in the boiled extract were absorbed on the anion exchange resin.

TABLE IV

Demonstration of the Requirement of the Three Components in the in vitro-Oxidation of Phthalate

Net O_2-uptake in μl.

Time in min.	Fr. I	Crude Fr. II1)	Fr. I+Boiled extract2)	Fr. I+Fr. II	Fr. I+Crude Fr. II
5	1.3	0.7	0.8	0.8	7.3
10	1.6	1.4	1.6	1.3	14.5
15	2.1	2.0	3.1	2.7	23.7
20	2.7	2.2	4.4	3.7	33.4

Net O_2-uptake in μl.

Time in min.	Fr. I+Fr. II+Boiled extract	Fr. I+Fr. II +DPN3)	Fr. I+Fr. II+ DPN+TPN4)	Fr. I+Fr. II+Boiled extract, without co-substrate
5	8.1	4.1	5.1	2.1
10	14.6	9.9	10.1	4.8
15	22.6	14.6	15.1	8.3
20	30.5	19.7	22.0	11.1

Each flask contained 10 μmoles of phthalate, 20 μmoles of ethanol as co-substrate, 0.25 ml. of $M/2.5$ phosphate buffer (pH 7.1), 10 μmoles of nicotinamide, 10 μ moles of cysteine. 0.25 ml. each of Fraction I, Fraction II, Crude Fraction II, Boiled extract and 2 μ moles of each of DPN and TPN were added to reaction mixtures. Final volume was made up with water to 5.0 ml. KOH-solution (10%), in the center well. Temperature, 20°.

1) Fraction II, which was not subjected to dialysis, was used as "Crude Fraction II".

2) A boiled extract of Crude Fraction II was used as "Boiled extract".

3), 4) DPN (90% pure) and TPN (approx. 65% pure) were obtained from General Biochemicals, Inc.

VIII. Intermediary Formation of Hydrogen Peroxide as Evidenced by the Inhibitory Effect of Catalase upon the Reaction—If, as we have postulated already, DPNH formed by the dehydrogenation of co-substrate would react with O_2 to form H_2O_2, and this H_2O_2 would then act as the direct oxidizing agent toward phthalate, the rate of the oxidation must be influenced by the presence of catalase in the reaction system. As stated already, Fraction II used in our experiments contained a certain amount of catalase. The activity of this catalase was reduced to about 1/3 of original strength, by centrifugation after acidifying the fraction to pH 4.4 with N-HCl at 0°–1°. Using the original and acid-treated samples of Fraction II, or by adding an excess of catalase (prepared from bovine liver) to the reaction system, the relation between the reaction rate and the catalase content in the reaction system was investigated. The experimental results given in Table V show that, in conformity with our expectation, the higher the content of catalase, the lower was the rate of oxidation of phthalate.

TABLE V

Relation between O_2-uptake and Catalase Content in the Reaction Mixture.

Net O_2-uptake in μl.

	Fr. II (0.25 ml)	Fr. II (0.75 ml)	Acid-treated Fr. II (0.25 ml)	Fr. II (0.25 ml) catalase in excess[2]
Relative catalase activity[1]	10	30	3	∞
Time in min.				
5	2.9	1.8	6.8	0.6
10	7.9	3.5	14.2	1.8
15	12.6	6.8	22.9	2.0
20	17.0	9.6	30.8	2.4

Each flask contained 0.25 ml. of Fraction I, 0.25 ml. of Boiled extract, 10 μmoles of phthalate. 20 μmoles of ethanol, 0.25 ml. of $M/2.5$ phosphate buffer (pH 7.1), 10 μmoles of nicotinamide, 10 μmoles of cysteine. Final volume was made up with water to 5.0 ml. KOH-solution (10%) in the center well. Temperature, 20°.

1) Catalase activity was measured manometrically at pH 7.1, 20°.

2) Concentrated bovine liver catalase was used.

IX. Effect of Various Substances—Phthalate oxidation occurring in the presence of co-substrate was affected by various substances in essentially the same way whether the reaction was brought about by intact cells or by cell-free enzyme system. (Compare the data presented in Table VI with those in Table III. The strong inhibition caused by cyanide and azide and monoiodoacetate and Hg^{++}-ion indicates that some heavy metal and sulfhydryl groups are involved in the reaction. It should be remarked that the oxidative reaction catalyzed by the cell-free enzyme, unlike that caused by intact cells, is not accompanied by CO_2-evolution. The metallo-enzyme and SH-enzyme which were assumed above may, therefore, be those participating in earlier stages of oxidation of phthalate, presumably preceeding the event of cleavage of aromatic ring.

As mentioned already, the oxidation of phthalate by hydrogen peroxide by intact cells was also strongly inhibited by azide, suggesting the participation of a peroxidase-like enzyme in the reaction. However, unlike phenylalanine hydroxylase (*8*) and aromatic ring-splitting enzyme, homogentisicase (*9*), the enzyme system of our organism is entirely refractory towards ferrous ion complexing agents such as α,α'-dipyridyl, versene and diethyldithiocarbamate, indicating that Fe^{++} ion may not be essential to the reaction.

X. Attempted Identification of Reaction Products—The fact that the oxidation of phthalate caused by the cell-free enzyme system occurs without liberation of CO_2 strongly points toward the occurrence of hydroxylation of benzene ring in the process. This inference was endorced by the observation that the reaction mixture gave a positive color reaction for phenolic compounds by nitroaniline test. Unfortunately, however, the substance could not be obtained in a quantity sufficient for further chemical identification.

TABLE VI

Effects of Inhibitors

Inhibitors	Final concentration	% inhibition
a) Cyanide	$M/1000$	100
Azide	,,	100
Fluoride	,,	-100 ~ -150
b) α,α'-dipyridyl	,,	0
Versene	,,	0
c) Monoiodoacetate	$M/2000$	100
Hg^{++}-ion	,,	100
d) Salicylate	$M/1000$	75

Easch flask contained 0.25 ml. of Fraction I, 0.25 ml. of Boiled extract, 10 μmoles of phthalate, 20 μmoles of ethanol, 0.25 ml. of $M/2.5$ phosphhate buffer (pH 7.1), 10 μmoles each of nicotinamide and cysteine, and inhibitors. Total volume, 5.0 ml. KOH-solution (10%) in the center well. Temperature, 20°.

As probable hydroxylated intermediates from phthalate one may envisage 4- and 3-hydroxyphthalates. These substances were found to be oxidized by the enzyme system (Fraction I and Fraction II, DPN and co-substrate) with a rate (in terms of O_2-uptake) that was about 2/3 of that observed when phthalate was used as substrate.

DISCUSSION

We believe that all the data described above may be explained on the basis of the reaction scheme which was presented in the introductory note of this paper. Biochemical oxidations requiring, as in the present case, the presence of reduced pyridine nucleotides and molecular oxygen have also been reported by some workers. For instance, Hayano *et al.* (*10*) and Grant *et al.* (*11*) observed that the oxidation of 11-deoxycorticosterone to 11-β-hydroxycorticosterone by adrenal extracts required TPNH and O_2, and according to Mitoma *et al.* (*12*) the same was true of the oxidation caused by microsomal particulates of certain aromatic compounds to their hydroxylated derivatives. It was also shown that DPNH and O_2 are involved in the oxidative cleavage of imidazole ring (*13*) and in the hydroxylation of phenylalanine to tyrosine (*6*). The peculiar fact that in all these cases the presence of *reduced* pyridine nucleotide is required for the occurrence of an *oxidative* process may receive a rational explanation when one assume, as we did in our hypothesis, that in the course of reaction the reduced pyridine nucleotide be oxidized by molecular oxygen – conceivably via the autoxidation of flavoprotein—to form hydrogen peroxide which functions as the virtual oxidant. In the processes studied by other workers, however, the attempts to obtain evidence in support of this hypothesis by several means—*i.e.*, (i) by the addition of catalase which may reduce the rate of reaction, or (ii) by adding, instead of reduced pyridine nucleotide, H_2O_2-generating systems such as glucose oxidase, D-amino acid oxidase, xanthine oxidase, uricase, *etc.* together with their respective substrates (*8*, *14*)—all proved to be unsuccessful, whereas it was possible in the present study not only to demonstrate directly the participation of H_2O_2, but also to adduce evidence of intermediary formation of H_2O_2 by the trapping method using catalase and methanol.

In our experiment the reaction system catalyzing the phthalate oxidation could be separated at least into three components: Fraction I, Fraction II and DPN. Several DPN-linked dehydrogenases and reduced pyridine nucleotide which are indispensable for the reaction were shown to be contained in Fraction II. As mentioned already, the crude preparation of Fraction II (before being subjected to dialysis) seems to contain, besides DPNH, some unknown cofactor or accelerator which could not be replaced by TPNH or metal ions. As for the enzyme system which catalyzes the oxidation of DPNH by molecular oxygen, it is not yet clear whether it is contained in Fraction I or Fraction II. Neither is it clear in what fraction the enzyme catalyzing the oxidation of phthalate by H_2O_2 is contained.

The fact that phthalate oxidation by hydrogen peroxide was strongly inhibited by azide may suggest that the last mentioned enzyme is a metalloenzyme having a peroxidase-like activity. It should be remarked, however, that the enzyme in question is, unlike phenylalanine hydroxylase and aromatic ring-splitting enzyme, homogentisicase (*9*), quite refractory towards the action of various ferrous ion complexing agents such as versene, α,α'-dipyridyl and diethyldithiocarbamate. The participation of ferrous ion which is known to accelerate the non-enzymatic hydroxylation by H_2O_2 of some aromatic compounds (*15*), may be exclude in the reaction of phthalate oxidation. (It was also ascertained that no phenolic compound was formed after incubating H_2O_2 (M/500), Fe^{++} (M/1000) and phthalate (M/1000) in our experiment.)

We have observed that the phthalate oxidation catalyzed by the cell-free enzyme system occurred without liberation of CO_2, while that brought about by intact cells was accompanied by CO_2-evolution. The RQ-value in the latter case was 0.94, a little lower than the theoretical value 1.07 calculated on the assumption of complete oxidation of the substrate. In the case of cell-free enzyme system the formation of some phenolic compound was revealed by a color reaction. Although the compound has not yet been identified, it may be certain that in intact cells phthalate is subjected to oxidative breakdown via some hydroxylated intermediates. The fact that 4- and 3-hydroxyphthalates were oxidized much more slowly than phthalate suggests that they may not be the intermediates in question. More probable may be the intermediate formation of 4,5-dihydroxyphthalate which was revealed by Evans (1) to accumulate in the medium in earlier stages of phthalate culture of some soil bacterium.

SUMMARY

1. It was found that the yeast cells oxidize *o*-phthalate coupled with aerobic oxidation of various substances (such as formate, acetate, lactate, ethanol, formaldehyde, vanillin, *etc.*) whose dehydrogenation is known to be catalyzed by DPN-linked dehydrogenases contained in yeast cells. Per mole of phthalate oxidized, 6.7 moles of oxygen were consumed and 6.3 moles of carbon dioxide were evolved.

2. It was revealed that a similar phenomenon could be brought about also by a cell-free preparation consisting of DPN, a labile enzymatic fraction, and a relatively stable fraction consisting various DPN-linked dehydrogenases as well as catalase. In this case, however, no liberation of CO_2 occurred and only 1 mole of O_2 was absorbed per mole of phthalate consumed. Nitroaniline test showed that some phenolic compound(s) was formed as the product of phthalate oxidation.

3. Using the cell-free preparation it was demonstrated that the coupled oxidation of phthalate is inhibited by the addition in excess of catalase. The inhibition of the same process was also brought about by the addition of methanol, which is known to cause, in the presence of catalase and hydrogen peroxide, the trapping of hydrogen peroxide. It was also shown that in the presence of hydrogen peroxide, phthalate is oxidized without being coupled with the oxidation of DPN-linked substrates.

4. Based on these observation it was inferred that in the coupled oxidation of phthalate, there occurs an intermediate formation of H_2O_2, which may function—in the presence of some peroxidase-like enzyme—as the virtual oxidant for phthalate. The state of affairs may reasonably be pictured as has been mentioned in p. 559, where AH_2 stands for the DPN-linked " co-substrate " such as formate or alcohol.

5. The reaction (both *in vivo* and *in vitro*) is strongly inhibited by cyanide and azide, but not by α,α'-dipyridyl and diethyldithiocarbamate. It is also inhibited by SH-inhibitors (*e.g.* monoiodoacetate and Hg^{++}-ion) and by some aromatic monocarboxylic acids (*e.g.* salicylic, benzoic, and phenylacetic acids). Fluoride inhibits the *in vivo*-reaction.

6. Besides phthalate the following substances were found to be oxidized in a similar manner, although at considerably lower rates than phthalate: 4- and 3-hydroxyphthalates, phenylglycine-*o*-carboxylate and *p*-hydroxybenzoate.

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REFERENCES

(*1*) Evans, W. C., *Biochem. J.*, **61**, (1956): *Proc. Biochem. Soc.*, **X**.

(*2*) Bray, H. G., and Thorpe, W. V., "Method of Biochemical Analysis," Vol. **1**, 46 (1954)

(*3*) Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, **147**, 415 (1943)

(*4*) Organic Synthesis, Collec., Vol. **II**, 597

(*5*) Fedorova, A. M., *Bull. Soc. Chim.*, **6**, 478 (1939)

(*6*) Gisvold, O., *J. Am. Pharm. Assoc.*, **31**, 202 (1942)

(*7*) Organic Synthesis, **56**, 408 (1951)

(*8*) Mitoma, C., *Arch. Biochem. Biophys.*, **60**, 476 (1956)

(*9*) Suda, M., Takeda, Y., Sujishi, K., and Takeda, T., *J. Biochem.*, **38**, 297 (1951)

(*10*) Hayano, M., and Dorfman, R. I., *J. Biol. Chem.*, **147**, 415 (1943)

(*11*) Grant, J. K., and Brownie, A. C., *Biochim. et Biophys. Acta*, **18**, 433 (1955)

(*12*) Mitoma, C., Posner, H. S., Reitz, H. C., and Udenfriend, S., *Arch. Biochem. Biophys.*, **61**, 431 (1956)

(*13*) Hayaishi, O., Tabor, H., and Hayaishi, T., *J. Am. Chem. Soc.*, **76**, 5570 (1954)

(*14*) Grant, J. K., Annual Reports on the Progress of Chemistry, *Chem. Soc.*, London, Vol. **LII**, 320 (1956)

(*15*) Udenfriend, S., Clark, C. T., Axelrod, J., and Brodie, B. B., *J. Biol. Chem.*, **208**, 731 (1954)

(*16*) Linnane, A. W., and Still, J. L., *Arch. Biochem. Biophys.*, **59**, 383 (1955)

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A NEW COLOUR REACTION OF SPHINGOSINE AND ITS USE FOR QUANTITATIVE ANALYSIS*

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Sphingosine is well known as a constituent of sphingolipids. The ninhydrin reaction has often been employed for its qualitative analysis, while its quantitative assay has been achieved by nitrogen determination of the chloroform soluble fraction of the sphingolipids hydrolysates. There is, however, a difficulty to be encountered in applying those methods to the specimen which is contaminated with certain water soluble bases. The present paper is concerned with a new colour reaction which can satisfactorily be used without any hinderance by the presence of such water soluble bases.

EXPERIMENTAL AND RESULTS

Part I. Qualitative Analysis

Materials (*i*) *Triacetylsphingosine and Sphingosine*—Triacetylsphingosine, in the form of needle-shaped crystal, was isolated by the method of Carter *et al.* (*1*) from the bovine spinal cord, having the following constants.

TABLE I

Physical and Chemical Constants of Triacetyl-sphingosine

	M.P. (°C)	$[\alpha]_D$ in chloroform	Iodine Value (Yasuda)
Carter *et al.*	101-102	-11.7	59.7 (cald.) 52.0
The present author	102-103	$\frac{-0.37\times100}{1\times3.2}=-11.56$	40.0

Sphingosine was obtained from 100 mg. of triacetylsphingosine, which was hydrolysed with 2.5 ml. of 0.5 N methanolic potassium hydroxide for 8 hours at 100° in a sealed tube. The hydrolysate was worked out to yield 73 mg. of amorphous sphingosine sulfate.

(*ii*) *N-Acetylsphingosine*—Sphingosine obtained from triacetylsphingosine was treated for N-acetylation in usual manner (*1*), and N-acetylsphingosine was separated from free sphingosine by paper electrophoresis. Material thus obtained was so minute that no attempt was made to determine its chemical constants. In Fig. 1A an example of electrophoresis of the crude N-acetylsphingosine is reproduced; there appear two non-wetting bands, B_1 and B_2, holding the original line in between, when the filter paper was washed with water. The band B_1 is reactive to fluorescein-sodium, ninhydrin and iodine, as seen in Fig. 1B, indicating that this is unreacted sphingosine. The B_2 band, on the other hand, is only reactive to iodine and is considered to be N-acetylsphingosine.

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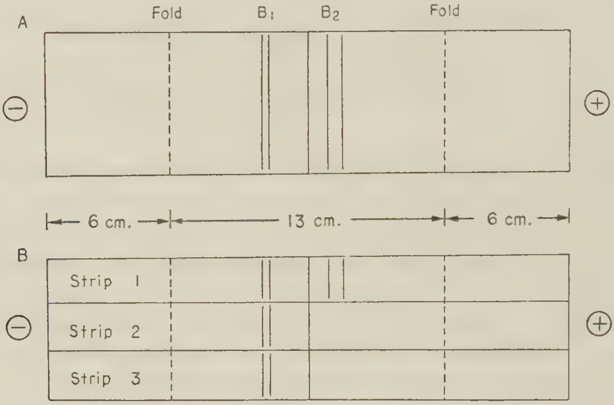

Fig. 1. Paper electrophoresis of the crude N-acetylsphingosine in an organic solvent mixture. Chloroform solution of the sample was applied to the central line and detected by dipping in the water for (A) and by spraying the iodine, ninhydrin and fluorescein-sodium solutions on strips 1, 2 and 3 respectively. B_1 is unreacted sphingosine and B_2 is N-acetylsphingosine. Paper electrophoresis was performed in an organic solvent mixture consisting of ethanol-pyridine-glacial acetic acid (1 : 1 : 1 by volume), pH 7.0, for 12 hours at room temperature. The field strength was 25 volts per cm. Toyo filter paper No. 51, 7×25 cm was used.

When the band B_2 was extracted with ethanol at 40°, and the solvent was removed by evaporation, there remained a waxy substance. By hydrolysis of this residue sphingosine could be obtained.

(*iii*) *Dihydrosphingosine*—The crude N-acetylsphingosine (100 mg.) was dissolved in ethanol and was hydrogenated over platinum oxide for 60 minutes under room temperature and normal pressure. Near the end of the reaction small particles appeared. Those particles were dissolved by heating. After removal of the catalyst by suction, the filtrate was allowed to stand overnight in refrigerator, and the precipitates were filtered. The yield was 810 mg. (90 per cent). Recrystallized in methanol, it gave N-acetyldihydrosphingosine with melting point 124-126° and iodine value 5.5. After 8 hours hydrolysis at 100° by 0.5 N methanolic potassium hydroxide, dihydrosphingosine was obtained as white powder; yield, 80 per cent.

Procedures of the Reaction and Detection Limit of Sphingosine or Its Derivatives—

On filter paper sphingosine reveals an orange red spot when sprayed with 0.25 per cent aqueous solution of fluorescein-sodium, and the colouration does not fade away by soaking in water. One and a half μg. of sphingosine and dihydrosphingosine can be detected by the present reaction, but N-acetylsphingosine, N-acetyldihydrosphingosine, triacetylsphingosine and triacetyldihydrosphingosine all show negative reaction.

Effect of Temperature, Hydrogen Ion Concentration and Concentration of the Reagent—In hot aqueous solution of fluorescein-sodium the colouration is quite insufficient. The coloured spot on a paper may be decolourized by soaking in water at 90°. The optimal concentration of fluorescein-sodium solution was found to be at about 0.25 per cent. With regard to the reaction of the reagent, the best results can only be expected at neutrality. If it be made acid, for example, with acetic acid, some fluorescein would be set free, making the reaction a complete failure; or when it be made alkaline (pH 7.4–10.0) by Na_2CO_3, the colouration would become less and less with increasing pH, and there would be no colouration whatever at pH 10.0. Colouration produced at neutrality can readily be changed to yellow or completely decoloured in acid or alkaline solution.

Detection of Sphingosine in Certain Synthetic Mixture with Lecithin and Cephalin

(*i*) *Sphingosine Admixed with the Phosphatides*—Four admixtures of the compositions shown in Table II were prepared and those were directly tested for sphingosine on the filter paper. The presence at the least of 15 μg. and in the proportion more than 24 per cent of sphingosine was required to be definitely recognized.

TABLE II

Detection of Sphingosine in the Mixture with Phosphatides

Composition			Result
Lecithin and cephalin (μg.)	Sphingosine (μg.)	(%)	
250	15	(6)	−
125	15	(12)	±
63	15	(24)	+
5	5	(50)	−

When this mixture was subjected to paper electrophoresis, employing the same organic solvent as used in the case just referred above, it was possible to detect the presence of 7.5 μg. (or in total composition 3 per cent) of sphingosine. The following figure is the reproduction of an example from such instances.

(*ii*) *Sphingolipids, Lecithin and Cephalin Mixture*—A mixture of 10 mg. of sphingolipids with 20 mg. each of lecithin and cephalin was hydrolysed with 5 per cent methanolic H_2SO_4 in a closed glass tube at 100° for 6 hours. After the hydrolysis, the content of the tube, still being acid, was extracted with ether, so as to remove fatty acids and their methylesters. The residue was made up to 2 ml. Ten μl. of this solution was used for the paper electrophoresis--conditions: acetate buffer pH 6.2, ionic strength: 0.16 and field strength: 10 volts per cm. The paper was treated with the fluorescein-sodium solution. The presence of sphingosine was indicated by orange red colouration of the original spot. Ten μl. of the specimen corresponds to 50 μg. of sphingolipids, so one can detect a presence of 50 μg. of sphingolipids by this procedure.

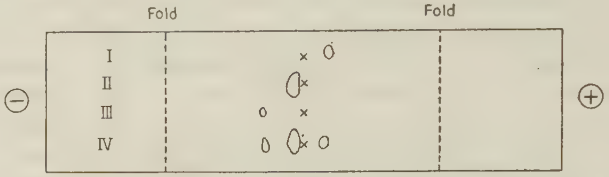

FIG. 2. Paper electrophoresis of lecithin, cephalin and sphingosine mixture. I: Lecithin 25 μg. II: Cephalin 225 μg. III: Sphingosine 7.5 μg. IV: Admixture of I, II and III. Spray reagent, aqueous solution of 0.25% fluorescein-sodium. Other conditions, the same as those of Fig. 1. Lecithin and cephalin appear as non-wetting spots and sphingosine as the specific orange red spot.

Substances Positive to the Present Reaction—There are a number of substances which show positive reaction to the fluorescein-sodium test; some are fat soluble bases like sphingosine and others are found among water soluble bases. Several examples of the latter group are shown in the following table.

TABLE III

Water soluble Bases and Their Relation to the Present Reaction

Substance	Reactivity	Liminal quantity (μg.)
Adrenalin	+	25
Agmatin H_2SO_4	–	
Aniline	–	
Arginine HCl	+	53
Betaine HCl	+	5
Choline HCl	–	
Diphenylamine	–	
Ethanolamine	–	
Histidine HCl	+	75
Lysine HCl	–	
Methylamine	+	6
Trimethylamine	+	30

The colours produced by such water soluble bases are, however, radically different in tint from that of sphingosine and its derivatives, and they can easily be erased completely by washing with water. Such differences in colouration make sphingosine and the allied substances easily differential from the other water soluble bases.

Part II. Quantitative Analysis

The analysis was performed densitometrically in the following way. Ethanol solution of sphingosine of 8 μl. was applied to the filter paper (Toyo paper No. 51, 3×15 cm.). After drying this was immersed for 30 seconds in 0.25 per cent fluorescein-sodium solution, followed by washing with running water for 20–30 minutes, dried over another filter paper, and lastly impregnated thoroughly with paraffin (melting point 50–52°) at 70° for 20–30 seconds. The optical density was measured on a spectrophotodensitometer (Hitachi)—slit width: 2 mm., and wavelength: 510 mμ. That the optical density thus measured is proportional to the amount of sphingosine is clearly seen in the calibration chart shown in Fig. 3.

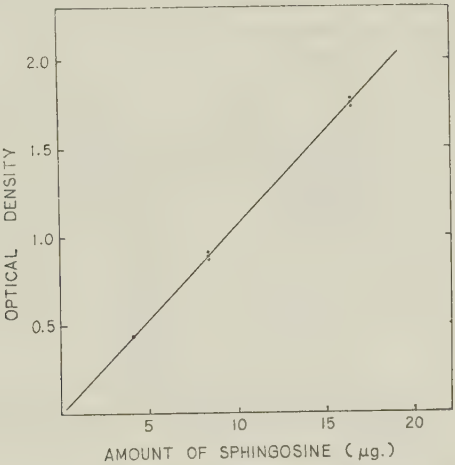

Fig. 3. Relation between the amount of sphingosine and optical density. Eight μl. of sphingosine in ethanol, applied to paper strip (Toyo Paper No. 51, 3×15 cm.); Colouration, by aqueous solution of 0.25% fluorescein-sodium; After rinsing in water, given paraffin coating; Measured on a spectrophotodensitometer (Hitachi) at the wavelength of 510 mμ.

With regard to the concentration of fluorescein-sodium, too high a concentration requires extra-long time for rinsing with water, and conversely too low a concentration makes the colouration insufficient. As the examples presented in Table IV indicate, there is hardly any difference between the tints when two concentration of 0.25 per cent and 2 per cent were employed—the duration of rinsing being 20–30 minutes.

The time allowed for rinsing, 20 to 30 minutes, was necessary to wash away the last trace of fluorescein-sodium, so as to keep the general background clear. An insufficient rinsing may cause trouble, making a sort of haloformation around the colour. The following figures may justify the above statement-between 20 and 30 minutes no alteration in tint could be detected.

The paraffin impregnation or coating process has more favourable properties over the use of liquid paraffin, glycerol or others, in that it has a

TABLE IV

Grade of Colouration and Fluorescein-sodium Concentration

% / μg.	$\sum$ E	
	0.25	2
16	1.39	1.46
8	0.70	0.70
4	0.38	0.33

Note: $\sum$ E denotes the total sum of extinction, % fluorescein-sodium concentration and μg. the amount of sphingosine.

TABLE V

Duration of Rinsing and Colouration

(Sphingosine: about 9 μg.)

Duration (Minutes)	$\sum$ E
10	0.892
20	0.860
30	0.867
40	0.850

Note: $\sum$ E denotes the total sum of extinction.

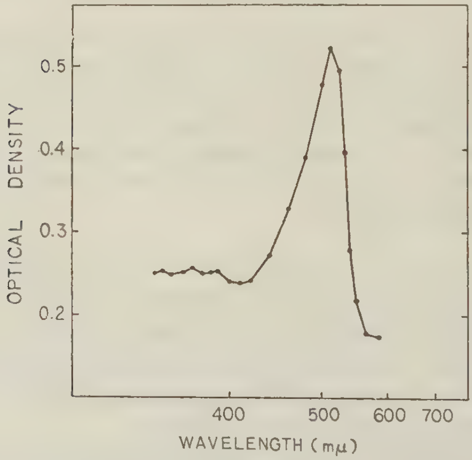

FIG. 4. Absorption curve of colour spot. Measured on the same conditions as Fig. 3.

higher transparency and a lesser tendency of forming the halo. Neverthless it requires certain precautions to get the optically homogeneous coating, not to mention of 'bubble formation'. Therefore, solid paraffin of low melting point (50–52°) was used at the temperature of about 70°. These two factors secure the uniform coating, free from the bubble nuisance. The relatively low temperature and the short period of immersion are good measures against a risk of discolouration, which otherwise inevitably occurs, if a paraffin of higher melting point be employed (consequently a much higher temperature of immersion, say 90° or so has to be used). The strip thus prepared remained colour-unchanged and stable over an hour with the absorption maximum at 510 $m\mu$. An example of the absorption curve of the colour spot is given in Fig. 4.

DISCUSSION

The present reaction is a micro-method essensial for the distinction of fat soluble bases, like sphingosine, *etc.*, from water soluble bases, and is more reliable than ninhydrin reaction which is reactive to both groups. So that whenever a hydrolysate shows a positive reactivity to the present test, the presence of sphingolipids should first be suspected. On the other hand, there is the method originated by McKibbin and Taylor (2) for the quantitative analysis of sphingosine, in which nitrogen of chloroform soluble bases is determined. But in this method a possibility of contamination by water soluble bases such as amines can not be excluded.

With respect to the mode of action of the test, among the 4 active groups of sphingosine—two hydroxyls, one amino group and one double bond—the free amino radical is absolutely indispensable. But it must be remembered that it is not the case that all the substances having amino radical should give positive reaction to the present test. On the other hand, fluorescein is a hardly soluble acid dye in water and becomes soluble in the form of sodium-salt. So the first stage of the reaction seems to be the combination of the amino group of sphingosine and carboxy group of fluorescein, resulting in the formation of coloured matters. In the second stage, the rinsing with water would dissolve away all soluble colours, leaving insoluble portion as the coloured spot.

Another acid dye, eosin, in ethanol when similarly employed, produced a red spot. If the comparison be made between eosin, free fluorescein and fluorescein-sodium for the merit as the colouration agent, fluorescein-sodium stands on the top, in that its detection power is the highest, it leaves the background colourless, and is positively active to either sphingosine or sphingosine sulfate.

SUMMARY

1. Sphingosine reacts with fluorescein-sodium on filter paper and gives specific orange red colour spot. The detection limit of the reaction is about 1.5 μg.

2. The use of the reaction for quantitative analysis, densitometrically performed, can be employed within the range of 4–16 μg.

The author wishes to thank Prof. S. Akashi, for his interest and encouragements during the present work.

REFERENCES

(*1*) Carter, H. E., Norris, W. E., Glick, F. J., Phillips, G. E., and Harris, R., *J. Biol. Chem.*, **170**, 269 (1947)
(*2*) McKibbin, J. M., and Taylor, W. E., *J. Biol. Chem.*, **178**, 29 (1949)

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BIOCHEMICAL STUDIES ON THE FORMATION OF THE SILKPROTEIN

VI. CONVERSION OF SERINE TO GLYCINE IN THE SILKWORM LARVA

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In mammals, the conversion of serine to glycine has been clarified by many research workers, but little is known of this conversion in insects, including silkworms. In silkworms, glycine is one of the main constituent amino acids (33 per cent) of the silkproteins produced by silkworms. The findings, therefore, of precursors of glycine synthesis seem to be of special importance in relation to the formation of the silk. It has already shown that one of the most important precursors of glycine synthesis in the silkworm larva is glyoxylic acid (*1*, *2*).

The current work was carried out, using L-serine all labeled with C^{14}, to show whether one of the precursors of glycine synthesis in the silkworm is serine. C^{14} labeled serine was given *per os* to silkworms, glycine was isolated from the silkprotein produced by these silkworms, and its radioactivity was measured. It was shown that serine is also one of the most important precursors of glycine synthesis in the silkworm larva.

EXPERIMENT

Administration of C^{14}-Serine to the Silkworms—L-Serine all labeled with C^{14} (13.46 mc per m mole was obtained from the Radiochemical Centre, Amersham, England. A half μc of radioactive serine per worm was given *per os* to the silkworms, Nichi 122×Si 115, at the fourth day of the fifth instar. These silkworms were reared on mulberry leaves until they produced cocoons.

Isolation of Glycine, Alanine, and Tyrosine in Fibroin—The separation of fibroin and sericin from cocoon fibres was carried out by procedures described in a previous paper (*3*). Two gm. of the fibroin was hydrolyzed with 6 ml. of concentrated hydrochloric acid for 10 hours. Tyrosine was isolated from the hydrolysate by procedures described by Abderhalden. Glycine and alanine were removed from the mother liquid respectively as a salt of 5-nitronaphthalene-1-sulfonate or as a salt of azobenzene-*p*-sulfonate, and then glycine and alanine were separated from their salts, following procedures described in a previous paper (*3*). Glycine and alanine were recrystallized respectively from water by repeated addition of absolute ethanol until their specific activities became constant. Tyrosine was recrystallized from hot water. The chemical purity of these isolated amino acids was proved by paper chromatography.

RESULTS AND DISCUSSION

The radioactivities of the amino acids isolated from cocoon fibres are shown in Table I. The radioactivity was found in glycine and alanine, but the specific activity of glycine was especially high. The radioactive glycine was degraded by steps according to the procedure described by Vernon *et al.* (*4*). Table II shows that the glycine molecule has higher labelling both in the 1 C and the 2 C position.

TABLE I

Radioactivity of the Amino Acids Isolated from the Cocoon Fibres Produced by the Silkworms given L*-Serine all labeled with* C^{14} *at the Fourth Day of the Fifth Instar*

Amino Acids	Radioactivity	Amounts of Amino Acids obtained from 2 gm. of Fibroin
Glycine	71550 c.p.m. per m mole	272.6 mg.
Alanine	15219 ,, ,, ,,	64.2 mg.
Tyrosine	0 ,, ,, ,,	24.0 mg.

TABLE II

Distribution of C^{14} *in Glycine Molecule*

Position in Molecule	Radioactivity
C-1 (-COOH)	35460 c.p.m. per m mole
C-2 (CH_2NH_2-)	27156 ,, ,, ,,

From these results, it was found that glycine in the silkworm larva is synthesized from serine.

As it has been elucidated recently that serine in the silkworm is synthesized from glycine (*5*, *6*), these facts seem to suggest that glycine and serine convert each other in the silkworm larva.

As stated also, in the previous paper (*1*), it has been pointed out that glyoxylic acid is one of the most important precursors of glycine synthesis in the silkworm larva. It will, therefore, be concluded that the most important precursors in the glycine synthesis of the silkworm larva are glyoxylic acid and serine. There are two pathways at least in the glycine synthesis of the silkworm larva; one of them is the pathway of glycine synthesized *via* serine, and the other is *via* glyoxylic acid. It will be discussed in thefollowing paper which is the main pathway of glycine synthesis of silkworms.

In silkworms, Nagayama *et al.* (*7*) recently insisted that glycine is synthesized from aminomalonate, and Bricteux-Grégoire (*8*) also pointed out recently that carbon of formate is utilized for glycine synthesis in the silkworm.

It was shown in this paper that serine is used for alanine synthesis in silkworms.

SUMMARY

1. The current work was carried out, using L-serine all labeled with C^{14}, to examine whether serine is utilized for the synthesis of glycine in the silk by *Bombyx mori*.

2. The isotope (C^{14}) of the L-serine all labeled with C^{14} given to the silkworms appeared in both the 1 C and the 2 C position of the glycine isolated from the fibroin produced by these silkworms.

3. These facts seem to suggest that the action which produces glycine from serine is of importance in the synthesis of the glycine of the silk in addition to the glycine formation by transamination from glyoxylic acid in the silkworm.

REFERENCES

(*1*) Fukuda, T., and Hayashi, T., *J. Biochem.*, **45**, 469 (1958)
(*2*) Koide, H., Shishido, T., Nagayama, H., and Shimura, K., *J. Agr. Chem. Soc. Japan*, **30**, 283 (1956)
(*3*) Fukuda, T., *J. Biochem.*, **43**, 137 (1956)
(*4*) Vernon, L. P., and Arronoff, S., *Arch. Biochem.*, **29**, 179 (1950)
(*5*) Fukuda, T., "The first Congress on Isotopes" (Japan), p. 528 (1956), Fukuda, T., *J. Biochem* (in press)
(*6*) Bricteux-Grégoire, S., Verly, W. G., and Florkin, M., *Nature*, **182**, 1515 (1958)
(*7*) Nagayama, H., Muramatsu, M., and Shimura, K., *Nature*, **181**, 417 (1958)
(*8*) Bricteux-Grégoire, S., and Verly, W. G., *Nature*, **182**, 1517 (1958)

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PHOSPHORUS METABOLISM OF FUNGAL CELLS

II. SOME PROPERTIES OF THE OXIDATIVE PHOSPHORYLATION SYSTEM OF MITOCHONDRIA FROM ASPERGILLUS ORYZAE

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In the preceding paper (*1*), it was reported that in mitochondrial particles isolated from mycelial mats of *Aspergillus oryzae* there was oxidative phosphorylation of succinate.

Since the P/O ratio was generally between 1.0 and 1.6, it was assumed that the mitochondrial preparation obtained with sucrose containing EDTA* and citrate was intact. Moreover mitochondrial phosphorylation was inhibited by antimycin A which inhibited the oxidative activity. It was also shown that the preparation was apparently less stable than that from animals since mitochondria prepared in the absence of citrate were rapidly aged and addition of nucleotide complex from the mycelial mats of this fungus promoted phosphorylation.

In this report, it is shown that the mitochondrial preparation could scarcely if at all utilize substrates other than succinate. The phosphorylative activity of the preparation was increased by cytochrome c, serum albumin and the nucleotide complex. The nucleotide complex seemed to stimulate as well as protect the phosphorylative activity, though its chemical nature is not yet known.

MATERIALS AND METHODS

Preparation of mitochondria, hexokinase, cytochrome c, DPN and the nucleotide complex, and the determination of oxidative phosphorylation were as described previously (*1*). Glutamic dehydrogenase was prepared by the method of Kubo *et al.* from hog liver (*2*). Nucleotides were separated chromatographically by the method of Potter *et al.* (*3*). Co A was determined by acetylation of *p*-aminoazobenzene (*4*) or of sulfanilamide (*5*).

RESULTS AND DISCUSSION

Substrates for Oxidative Phosphorylation—In the previous paper (*1*) the oxidation of succinate was described. Attempts were made to use other oxidizable substrates such as isocitrate, lactate, malate, pyruvate, α-ketoglutarate, β-hydroxybutyrate and ethanol. Malate increased phosphate uptake, but was scarcely oxidized. The P/O ratio was therefore more than that with succinate (Table I).

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* Abbreviations are used in this paper: MP, monophosphate; DP, diphosphate; TP, triphosphate; A, adenosine; G, guanosine; U, uridine; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; Co A, coenzyme A; EDTA, ethylenediaminetetracetate.

TABLE I

Substrates for Oxidative Phosphorylation

Preparation medium of mitochondria	Substrate added	Phosphate uptake (μ moles)	Oxygen consumption (μ atoms)	P/O ratio
A	None	0.11	0.21	—
	Isocitrate	0.11	0.17	—
	Lactate	0.09	0.31	—
	Ethanol	0.14	0.30	—
B	None	0.78	0.66	—
	Succinate	2.59	1.60	1.62
	Malate	1.73	0.97	(1.78)
	Pyruvate	0.68	0.72	—
	α-ketoglutarate	0.93	0,80	—
	β-hydroxy-butyrate	0.78	0.66	—

Reaction system in Warburg vessel was as follows. Main chamber; 10 μ moles *i*P (radioactivity of *i*P^{32} is 5,000～10,000 c.p.m. per μ mole), 5 μ moles $MgCl_2$, 2 μ moles ATP, 10 μ moles KF, 20 μ moles glucose, hexokinase (enough to react with 5 μ moles of ATP for 10 minutes under these condition), DPN, 0.5 mg. per vessel, 0.1 ml. of nucleotide complex equivalent to 2 g. of mat wet weight, 20 μ moles of substrate, and water to 0.8 ml. Side arm; 0.2 ml. of mitochondrial suspension (equivalent to 4 g, wet weight). Centre well; 0.1 ml. of 40% KOH. TPN is present in the nucleotide complex. Preparation medium; A, sucrose 0.5 *M*, EDTA 0.01 *M*, pH 7.2, B, A plus citrate 0.006 *M*, pH 7.2. Temperature, 25°. Reaction period, 30 minutes.

According to the report of Utter *et al.* (*6*), yeast mitochondria disrupted in NaCl-EDTA can oxidize succinate and lactate, but not malate and β-hydroxybutyrate. Moreover, the preparation could oxidize isocitrate with accompanying phosphorylation on addition of protein factors. These protein factors, released from the mitochondria, were precipitated with ammonium sulfate and dialysed before use.

Oxidation of externally added DPN by the mitochondrial preparation from *Aspergillus oryzae* was studied. A glutamic dehydrogenase system from hog liver was added with the DPN to regenerate DPNH. As shown in Table II, DPNH affects oxidation slightly but has no effect on phosphorylation. This is consistent with the reports of other investigators (*6*, *7*). A protein fraction was prepared from the supernatant by precipitation with ammonium sulfate (80 per cent saturation) and was dialysed against cold water. It contained Mg-activated ATP-ase and glucose-6-phosphate phosphatase. This preparation suppressed phosphorylation, probably because of its phosphatases. The data is shown in Table III.

TABLE II

Glutamic Dehydrogenase, Oxidation of DPNH and Phosphorylation

Materials added	Phosphate uptake (μmoles)	Oxygen consumption (μatoms)	P/O ratio
None	0.34	0.48	(0.71)
Glutamate (20 μ moles)	0.78	0.75	1.04
Glutamate+glutamic dehydrogenase	0.78	0.96	0.80

Experimental conditions as in Table I.
Preparation medium; B.

TABLE III

Effect of Supernatant Protein Solution on Oxidative Phosphorylation

Materials added	Phosphate uptake (μ moles)	Oxygen consumption (μ atoms)	P/O ratio
None	0.34	0.26	—
Glutamate (20 μ moles)	0.74	0.58	1.28
,, +supernatant protein	0.52	0.45	1.15
,, +nucleotide complex	0.84	0.64	1.31
,, +supernatant protein+nucleotide complex	0.51	0.54	0.80

Experimental conditions as in Table I. Preparation medium; B.

When the mitochondria were suspended in hypotonic sucrose (*7*) oxidation and phosphorylation were not increased by ascorbate and cytochrome c (Table IV). These results do not indicate a deficiency of cytochrome oxidase, because, as with mitochondria partially damaged by various detergents (*9*), the P/O ratio with succinate is usually higher than 1. Therefore this mitochondrial preparation seems to have little pyridine coenzyme-dehydrogenase or system other than the cytochrome system which can oxidize DPNH.

Protective Effect of Some Proteins on Oxidative Phosphorylation—It has been shown that yeast mitochondria (*6*) and housefly mitochondria (*10*) are protected from aging by serum albumin. Both oxidation and phosphorylation of our fungal preparation were much increased by serum albumin, as shown in Fig. 1 though the P/O ratio was scarcely altered.

As shown in Fig. 2 a, increase in oxygen uptake was proportional to the concentration of cytochrome c, but phosphate uptake gradually decreased to a certain concentration of cytochrome c (3×10^{-5} *M*) and then increased in parallel with the increase of oxidation. To clarify this effect of cytochrome

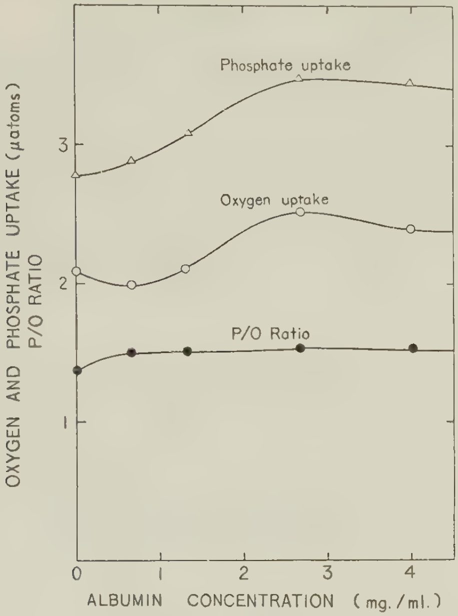

Fig. 1. Effect of serum albumin on oxidative phosphorylation. Experimental conditions as in Table I. Preparation medium: B.

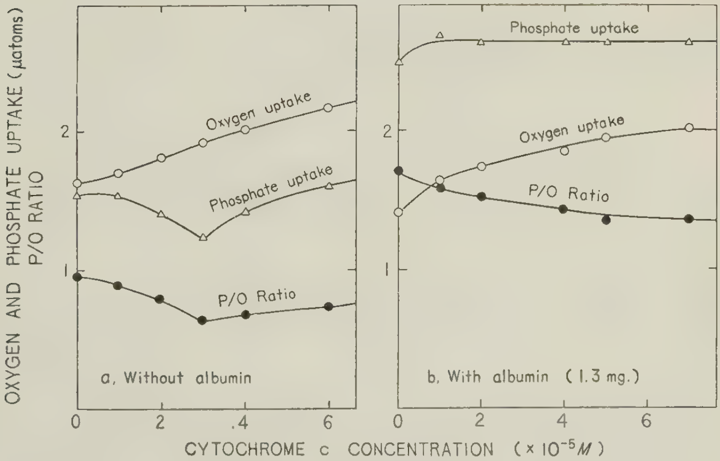

Fig. 2. Effects of combination of cytochrome c and serum albumin on oxidative phosphorylation. Experimental conditions as in Table I. Preparation medium: B.

c, 1.3 mg. of serum albumin was added with various amounts of cytochrome

c. As shown in Fig. 2 b, the decrease in phosphorylation observed in the experiment was prevented by addition of cytochrome c. The phosphorylative activity, like the P/O ratio, was very high.

Therefore, the stimulation of phosphorylation caused by a high concentration of cytochrome c (more than $4 \times 10^{-5}\,M$) is not a specific effect, but a general action of a protein.

Effect of Nucleotide complex from Aspergillus oryzae on the Oxidative Phosphorylation—As described previously (*1*), a nucleotide complex prepared from mycelial mats of this fungus by charcoal adsorption and elution, increases the P/O ratio, generally stimulates phosphorylation, but no effect on oxidation. The same effect was observed by with DPN, as described by Ernster *et al.* (*11*). The effect is greater on combination of the nucleotide complex with DPN.

To see whether the nucleotide complex increases the P/O ratio by preventing aging or whether it restores the phosphorylative activity lost by aging, the following experiments were made. When nucleotide complex was preincubated in the side arm of a Warburg vessel with mitochondria prepared without citrate. Phosphorylative activity was unchanged though the control which was preincubated without nucleotide complex lost almost all its activity (Table IV). Therefore the nucleotide complex may prevent aging of the mitochondria. However, this protection was not intensified by addition of DPN.

TABLE IV

Prevention of Aging by Nucleotide Complex

Materials added	Preincubation without nucleotide complex			Preincubation with nucleotide complex		
	Phosphate uptake (μ moles)	Oxygen consumption (μatoms)	P/O ratio	Phosphate uptake (μ moles)	Oxygen consumption (μatoms)	P/O ratio
None	0	0	—	0.01	0.41	—
Succinate (40 μmoles)	0.10	0.76	0.13	1.29	0.86	1.49
,, +DPN	0.11	0.89	0.12	1.29	0.89	1.45
,, +cytochrome c	0.15	1.03	0.15	0.87	1.33	0.65
,, +cytochrome c+DPN	0.12	0.82	0.15	1.60	1.21	1.32
Cytochrome c+ascorbate	0.02	0.94	0.02	0.04	1.34	0.03
Cytochrome c[1]	0.02	0.07	—	0.07	0	—
,, +ascorbate[1]	0.08	1.01	0.08	0.13	1.67	0.08

1) Mitochondria were suspended in 0.1 *M* sucrose. Other mitochondria were suspended in 0.3 *M* sucrose. Preincubation was carried out for 20 minutes at 25° with nucleotide complex or water. Other experimental conditions as in Table I. Preparation medium; A.

When the nucleotide complex was mixed with the mitochondria at the beginning of the reaction, an increase in the P/O ratio was seen (*1*). Therefore, may be not only a promoting but also a protecting effect on phosphorylation. To examine the chemical nature of the active material(s) in the nucleotide complex having protecting and promoting effects on phosphorylation, gradient anion exchange chromatography was used. The sample was put on a Dowex 1, ×2 column (formate form) and eluted with formic acid. It was rechromatographed, this time eluting with ammonium formate. The results are shown in Fig. 3. Enough eluate was collected to separate several fractions.

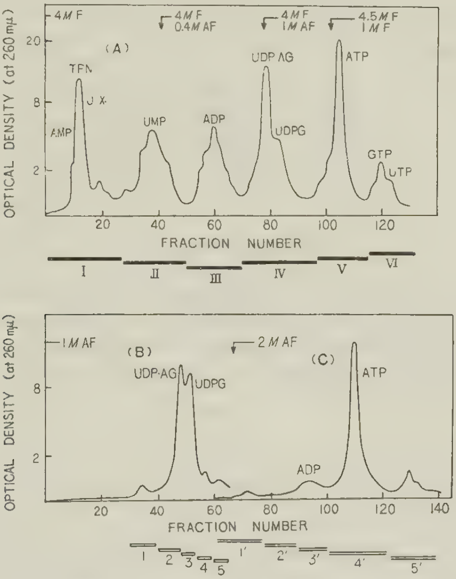

Fig. 3. Chromatography of nucleotide complex. (A) First chromatography. (B) Rechromatography of fraction IV. (C) Rechromatography of fraction V. See text and (*1*) for details.

These were treated by charcoal (*1*) to remove formate. Although there was some loss (10～20 per cent) in this charcoal treatment, it was available for qualitative test.

As shown in Table V, certain fractions of the first eluate prevented aging. For example an effect was seen with fractions containing UMP, ATP, a mixture of UTP and GTP, and UDP-compounds caused stimulation. In the eluate of the second chromatogram stimulation was caused by a fraction which was not UDP-acetylglucosamine or UDP-glucose or ADP, as shown in Table VI.

Simultaneously Co A assays were made on each fraction and it was shown that the active material was not Co A.

TABLE V

Effect of Whole and Fractionated Nucleotide Complex on Prevention of Aging and Stimulation of Oxidative Phosphorylation

Nucleotide complex or chromatographic fraction added	Prevention of aging (A)			Stimulation of phosphorylation (B)		
	Phosphate uptake (μ moles)	Oxygen consumption (μatoms)	P/O ratio	Phosphate uptake (μmoles)	Oxygen consumption (μatoms)	P/O ratio
None	0.27	0	—	0.60	0.38	1.58
Nucleotide complex	0.27	0.14	(1.93)			
Succinate (40 μ moles)	0.76	1.09	0.70	0.74	1.33	1.31
,, +nucleotide complex	1.21	0.95	1.27	1.82	1.25	1.45
,, +fraction I	0.64	0.95	0.67	1.64	1.29	1.27
,, +fraction II	0.97	0.97	1.00	1.78	1.33	1.34
,, +fraction III	0.84	0.95	0.88	2.04	1.40	1.45
,, +fraction IV	0.92	0.97	0.95	2.12	1.31	1.69
,, +fraction V	1.00	0.97	1.03	1.99	1.41	1.41
,, +fraction VI	1.01	1.01	1.00	1.78	1.50	1.19

(A); preparation medium A. (B); preparation medium B. Total nucleotide; (I) 36.0 (II) 28.1 (III) 27.0 (IV) 55.3 (V) 62.6 (VI) 15.6 μ moles. Co A; (I) 0 (II) 0 (III) 12.5 (IV) 72.0 (V) 30.0 (VI) 5.2 Lipmann units. Other experimental conditions as in Table I. For tests one fiftieth of each fraction was used.

TABLE VI

Protection of Oxidative Phosphorylation by Nucleotide Complex

Fraction No. of nucleotide	Total nucleotide (μmoles)	Co A (units)	Phosphate uptake (μmoles)	Oxygen consumption (μatoms)	P/O ratio
—	—	—	2.75	2.38	1.15
1	4.2	0	2.95	2.49	1.19
2	15.8	0	2.81	2.38	1.18
3	17.6	0	2.82	2.28	1.24
4	6.7	0	2.81	2.24	1.25
5	3.2	0	3.50	2.24	1.56
—	—	—	3.00	2.48	1.21
1′	1.1	0	3.48	2.40	1.45
2′	1.8	1.6	2.88	2.44	1.18
3′	3.2	3.6	3.15	2.66	1.18
4′	43.0	15.2	2.76	2.42	1.14
5′	4.8	8.4	2.82	2.45	1.15

The fraction Nos. correspond to those of Fig. 3. Other experimental conditions as in Table I. For each test one fifth of each fraction was used.

SUMMARY

Some properties of mitochondria from *Aspergillus oryzae* were studied. Only succinate dehydrogenase activity remained. Other dehydrogenases such as isocitric, lactic, pyruvic, α-ketoglutaric, β-hydroxybutyric, and the reduced DPN-oxidizing enzyme system were lost during preparation of the mitochondria.

Cytochrome c and serum albumin prevented loss of activity to a certain extent. A nucleotide complex prepared from the fungal mats prevented aging of the mitochondria. The active materials in this preparation are nucleotides commonly found in the cells. Stimulation of phosphorylation was also observed, but the action mechanism is still obscure.

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REFERENCES

(*1*) Iwasa, K., *J. Biochem*, **47**, 445 (1960)
(*2*) Kubo, H., Yamamoto, T., Iwatsubo, M., Watari, H., Soyama, T., Shiraishi, J., Sawada, S., and Kawashima, N., *Proc. Intern. Sym. Enz. Chem. Tokyo-Kyoto*, p. 345 (1957)
(*3*) Hurlbert, R. B., Schitz, H., Brumm, A. F., and Potter, V. R., *J. Biol. Chem.* **209**, 23 (1954)
(*4*) Handschumacher, R. E., Mueller, G. C., and Strong, F. M., *J. Biol. Chem.*, **189**, 335 (1951)
(*5*) Kaplan, N. O., and Lipmann, F., *J. Biol. Chem.*, **174**, 37 (1948)
(*6*) Utter, M. F., Keech, D. B., and Nossal, P. M., *Biochem. J.*, **68**, 431 (1958)
(*7*) Lehninger, A. L., *Harvey Lect.*, **49**, 176 (1950)
(*8*) Slater, E. C., *Nature*, **174**, 1143 (1954)
(*9*) Imamoto, F., *J. Biochem.*, **46**, 1023 (1959)
(*10*) Sacktor, B., *J. Biol. Chem.*, **233**, 1233 (1958)
(*11*) Lindberg, O., and Ernster, L., *Nature*, **173**, 1038 (1954)

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STUDIES ON CYTOCHROME C*

IV. MODE OF EXISTENCE OF NATIVE AND MODIFIED CYTOCHROME C

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Margoliash (*1*, *2*) has reported a method for the purification of cytochrome c by chromatography on a weakly acidic resin. Morrison, Estabrook and Stotz (*3*) reported that cytochrome c purified by this method to remove so-called modified cytochrome c, yielded two hemins when subjected to the silver treatment of Paul (*4*). Stotz *et al.* (*5*) suggested that these two kinds of cytochrome c fractionated by chromatography of the usual Keilin-Hartree preparation of cytochrome c (*6*) in its oxidized form on an Amberlite IRC-50 column might be derived from two species of cytochrome c_A and cytochrome c_B.

Henderson and Rawlinson (*7*) have measured oxidation-reduction potentials of cytochrome c's purified from heart muscles of horse and ox, and have reported that the normal redox-potential of highly purified cytochrome c is 0.255 V. at pH 6.4 and 25°, and of less pure preparations such as the Keilin-Hartree preparation is about 15 mV. higher, while those of the modified forms are 0.308 V.

Cytochrome c was crystallized from skeletal muscle of the king penguin by Bodo (*8*). Just afterwards, it was crystallized from several other sources by other workers, mostly in this laboratory (9-15).

Cytochrome c prepared from animal sources by the method of Keilin and Hartree (*6*) with trichloroacetic acid can be separated into more than two fractions on an Amberlite CG-50 column. The fractionated cytochrome c's have the same oxidized and reduced absorption spectra, and if assayed with Green brei and baker's yeast lactic dehydrogenase, the activities of the oxidized and reduced forms are almost the same (*14–17*). However, the fractionated cytochrome c's vary in susceptibility to digestion by some proteolytic enzymes. The larger the susceptibility, the higher the ascorbic acid oxidizing activity. Moreover, it has been shown that cytochrome c can be obtained as a single fraction on a resin-chromatogram with the least susceptibility, if all of the purification procedures are carried out at a low temperature and at neutrality. If rechromatographed after heating or treating at too acidic or too alkaline pH, the cytochrome c obtained by the mild purification procedure mentioned above, is altered to yield a sample similar to the fraction of the Keilin-Hartree preparation which has the strongest affinity for Amberlite IRC-50, shows the largest susceptibility and has the highest ascorbic acid oxidizing activity. The presence of trichloroacetic acid strongly accelerates this modification at an acidic pH and room temperature, but negligibly at a neutral pH and low temperature, when treated only for a few minutes. These findings indicate that the cytochromes suggested by Stotz *et al.* (*5*) to be c_A and c_B might correspond to a native cytochrome c and a modified cytochrome c. Suitable comparisons of various purification-procedures and of the properties of cytochrome c's fractionated by resin-chromatography with cytochrome c's artificially modified by fractionation, allowed the determination of which fraction is " experimentally native " cytochrome c (*15–18*). Cytochrome c is easily crystallized from the fraction, and it has been confirmed that this crystallizing procedure does not alter any of the properties of the cytochrome c mentioned above.

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In addition to the usual water-soluble cytochrome c, Widmer and Crane (*19*) have extracted a cytochrome c-like substance from animal mitochondria by an organic solvent, which they have called lipid cytochrome c.

This paper deals with some of the physicochemical differences between the native and modified cytochrome c's with special reference to their water-solubility. The results indicate that cytochrome c modified by acidic treatment exists as a dimer.

MATERIALS AND METHODS

Purification of Native Cytochrome c—Cytochrome c was purified from bovine heart muscle according to the mildest method established so far of Yamanaka, Mizushima, Nozaki, Horio and Okunuki (*17*). This method did not use trichloroacetic acid and was strictly limited to between pH 6 and 8 within which range no imidazole radical of cytochrome c is dissociated (*20*). The cytochrome c purified by this method moved on an Amberlite CG-50 (Type II) column as only one fraction (Fraction II), which was native cytochrome c adjudged experimentally (*15–18*). The native cytochrome c was crystallized in its reduced form from ammonium sulfate and stored in crystalline state in a refrigerator. Before use, it was recrystallized more than three times and then dialyzed in a cellophane sack against several volumes of an appropriate buffer for 72 hours in a refrigerator (4–5°) the outside buffer being changed several times. The reduced form was obtained from the oxidized form by addition of a minimal amount of sodium dithionite. Cytochrome c reduced by hydrogen gas in the presence of platinium asbestos gave the same results as that reduced with dithionite. The presence of traces of heavy metals was avoided during the experiments, since they notably catalyze the autoxidation of reduced cytochrome c.

Preparation of Modified Cytochrome c—The cytochrome c recrystallized more than three times was dissolved in water. After oxidation by a minimal amount of potassium ferricyanide, the dissolved sample was dialyzed overnight against several volumes of distilled water and diluted until its extinction at 280 mμ in a one-cm. cuvette reached approximately 10. One-tenth volume of 0.1 *M* trichloroacetic acid was added to the diluted cytochrome c solution which was then stored overnight at room temperature (10–20°).

Some of the cytochrome c became insoluble under these experimental conditions. To the solution, N NaOH was added dropwise in an ice bath until the insoluble cytochrome c was completely dissolved (about pH 9). The resulting solution was dialyzed overnight against several volumes 0.01 N NaCl in a refrigerator. The dialyzed cytochrome c was chromatographed on an Amberlite CG-50 column according to the method of Yamanaka *et al.* (*17*) and the fraction I was collected. After concentration by the adsorption-elution procedure, the sample (modified cytochrome c) was dialyzed against an appropriate buffer in the same way as for native cytochrome c. In other experiments, the modified cytochrome c was found to develop as a single chromatographic species by all the various elution methods tried (especially, by varying types and concentrations of buffer used, and flow rates).

Sedimentation—Sedimentation was measured with a Spinco Model E Ultracentrifuge. Runs were carried out at 59,780 r.p.m. ($259{,}700\times g$) at room temperature (about 20°). The average values of the initial and final temperature of the rotor were taken as the experimental temperatures. The sedimentation constants were calculated by the method of Svedberg and Pedersen (*21*).

Diffusion—Diffusion was studied by the Schlieren cylindrial lens-method using a Neurath type cell. The temperature of the thermostat was regulated within ±0.01° by the technique of Moriguchi (*22*), and measurements were carried out at 20°. The diffusion coefficients were calculated by three of Neurath's different methods (*23*), namely 1) the method of the inflexion point, 2) the method of successive analysis and 3) the area method.

Partial Specific Volume—The density of the solution and of the solvent was measured at 20±0.05° using an Ostwald type pycnometer with a capacity of about 3 ml.

Buffer—The buffer used in sedimentation and diffusion measurements was 0.02 M sodium phosphate buffer of pH 7.6 containing 0.01 M sodium chloride ($\mu=0.15$).

RESULTS

Sedimentation Constants of Native Ferro- and Ferri-cytochrome c and of Modified Ferri-cytochrome c—The sedimentation constants of native and modified cytochrome c prepared from bovine muscle are listed in Table I. With neither of the cytochrome c's were the constants notably dependent on the concentration in the range listed in Table I. The average values of the sedimentation constants at 20° in water ($S_{20,w}$) were calculated to be 1.87, 1.91 and 2.50 Svedberg units for native ferro-, native ferri- and modified ferri-cytochrome c, respectively. Sedimentation constants of the native cytochrome c can be regarded as being the same for the oxidized and reduced forms. With the use of the highly purified cytochrome c, Pedersen and Andersson (*24*) have reported that the $S_{20,w}$ is 1.91 Svedberg units, the same value as obtained in the present experiment for native cytochrome c. However, the sedimentation constant of the modified cytochrome c is notably larger than that of native cytochrome c, indicating that the modified cytochrome c exists in a state quite different from the native cytochrome c.

Diffusion Coefficients of Native Ferro- and Ferri-cytochrome c and of Modified Ferri-cytochrome c—Diffusion coefficients were calculated using the same precautions taken by Isemura and Fujita (*25*) in their work with Taka-amylase A. The diffusion coefficients were calculated by three different methods. The values thus obtained agreed well with each other, as shown in Table II. The diffusion coefficient was almost independent of the protein concentration used. The diffusion coefficients at 20° in water ($D_{20,w}$) were 12.9×10^{-7}, 13.2×10^{-7} and 8.5×10^{-7} cm.²/sec. on an average for native ferro-, native ferri- and modified ferri-cytochrome c, respectively.

TABLE I

Sedimentation Constants of Native and Modified Cytochrome c

The measurements were carried out at pH 7.6 in sodium phosphate buffer adjusted to an ionic strength of 0.15 with NaCl.

Native cytochrome c reduced form :

Protein conc. (g./100 ml.)	$S_{20,\omega}\times10^{13}$	$S_{20,\omega}$ average
0.27	1.91	1.87×10^{13}
0.34	1.89	
0.61	1.83	
0.78	1.85	

Native cytochrome c oxidized form :

Protein conc. (g./100 ml.)	$S_{20,\omega}\times10^{13}$	$S_{20,\omega}$ average
0.28	1.92	1.91×10^{13}
0.56	1.90	
0.80	1.93	
0.99	1.89	

Modified cytochrome c oxidized form :

Protein conc. (g./100 ml.)	$S_{20,\omega}\times10^{13}$	$S_{20,\omega}$ average
0.21	2.54	2.50×10^{13}
0.44	2.49	
0.71	2.51	
0.93	2.46	

With the use of a rather crude preparation of cytochrome c, Polson (*26*) has reported that the $D_{20,w}$ is 10×10^{-7} cm.²/sec.. As in the case of sedimentation, it is considered that the diffusion coefficient of cytochrome c may be changed by contamination of native with modified cytochrome c, since the value is notably smaller for the modified cytochrome c. It seems likely that Polson's value might have been lowered by the presence of a large amount of the modified cytochrome c which have been present as a result of using the classical drastic purification-procedure.

Partial Specific Volumes of Native Ferro- and *Ferri-cytochrome c and of Modified Ferri-cytochrome c*—The partial specific volume of native ferro- and ferri-cytochrome c and of modified ferri-cytochrome c were determined. These different kinds of cytochrome c's were found to have the same partial specific volume of 0.707. This value is in accord with the value reported by Theorell (*27*) for partially purified cytochrome c from bovine heart.

Molecular Weights of Native and Modified Cytochrome c—The molecular weights of the cytochrome c's were calculated from those of $S_{20,w}$, $D_{20,w}$ and partial specific volume in the usual manner. The molecular weight of native cytochrome c is 12,000 in both its oxidized and reduced form. This value corresponds to the minimal molecular weight of cytochrome c calculated on the basis of its iron content (0.47 per cent). On the other hand, the molecular weight of the modified cytochrome c in its oxidized form was 24,000. This value is twice that of native cytochrome c. From a simple consideration on the molecular weight and without consideration of any difference in secondary structure of the protein moiety (*15–18*), this indicates that the molecule of modified cytochrome c exists as a dimer of the native cytochrome c.

TABLE II

Diffusion Coefficients of Native and Modified Cytochrome c

The measurements were carried out at 20° in the same buffer as for Table I.

Native cytochrome c reduced form:

Protein conc. (g./100 ml.)	$D_{20,\omega} \times 10^{-7}$ cm.^{2}/sec.
0.29	12.8
0.33	13.0
0.60	12.9
0.79	13.0
	12.9 average

Native cytochrome c oxidized form:

Protein conc. (g./100 ml.)	$D_{20,\omega} \times 10^{-7}$ cm.^{2}/sec.
0.45	13.3
0.57	13.0
0.80	13.2
1.30	13.1
	13.2 average

Modified cytochrome c oxidized form:

Protein conc. (g./100 ml.)	$D_{20,\omega} \times 10^{-7}$ cm.^{2}/sec.
0.31	8.2
0.55	8.4
1.37	8.9
	8.5 average

DISCUSSION

The native cytochrome c in both its oxidized and reduced forms can be easily fractionated into two different forms by chromatography on an

Amberlite CG-50 column. These forms can be easily and completely separated from each other. The reduced forms of these compounds have less affinity for the resin than oxidized forms. On this resin, the modified cytochrome c prepared by the acidic treatment of native cytochrome c used in this paper, is difficult to elute by ordinary buffers. However, it is easily eluted by concentrated buffer, and shows only one chromatographic species. Therefore, the chromatographic species of cytochrome c may be notably changed by an alteration of the valency of heme-iron. This difference in the native cytochrome c should reflect principally ionic differences between the oxidized and reduced forms. In a similar way, the modified cytochrome c may differ to a remarkable extent from the native cytochrome c in its ionic state. Minakami *et al.* (*28*) reported that cytochrome c increases in redox-potential when digested by trypsin. It is therefore possible that these changes in redox-potentials of cytochrome c are caused by a certain changes in the protein-moiety as well as in heme-moiety. The native cytochrome c is hardly digested by trypsin in its reduced form but is easily digested in its oxidized form. Moreover, the modified cytochrome c is more susceptible to proteolytic enzymes in its oxidized form than the native cytochrome c (*15–18*). This suggests that some change of the protein-moiety of the native cytochrome c is coupled with a ferri-ferro interconvertion of heme-iron. However, this change has not yet been demonstrated physicochemically by such means as measurement of sedimentation constant, diffusion coefficient or partial specific volume.

On the other hand, Tuppy and Palèus (*29*) found that the cytochrome c digested by trypsin (tryptic cytochrome c) has a molecular weight of 2,000 at pH 2.3 and of 10,800 at pH 8.7. Their data indicate that tryptic cytochrome c molecules may form polymers at an alkaline pH. The presence of histidine can prevent this polymerization, possibly due to a conjugation of the tryptic cytochrome c with histidine. Theorell and Åkesson (*20*) also reported that at least one of the valencies of the heme-iron of cytochrome c is paired with the imidazole radical of the histidine of the heme-peptide of cytochrome c, and that this dissociates above and below pH 5.5∼8.5. When acid treatment is used in the purification procedure (treatment with trichloroacetic acid (*6*), extraction at an acidic pH with sulfric acid (*30*) or acetic acid (*31–35*), or adsorption on acidically buffered resin (*31–35*)), cytochrome c can be more or less separated, according to how drastic is the treatment used, into two chromatographic fractions consisting of a native and modified cytochrome c, or into a single chromatographic fraction consisting entirely of modified cytochrome c. However, when pH is kept at a neutral range (pH 6–8) throughout purification procedure, only one chromatographic species of cytochrome c results and this has been shown to be the native cytochrome c (*15–18*). These facts suggest that cytochrome c is easily modified in the pH range where histidine dissociates, but not at a neutral pH-range. Stotz *et al.*'s findings may support the possibility that at least one of the modifications of cytochrome c results from a change in the part of the molecule where the heme and heme-peptide are linked.

Cytochrome c modified by acidic treatment exists as a dimer. From the result of Tuppy and Palèus on tryptic cytochrome c, it seems that molecules of cytochrome c have a tendency to bind with each other in the pH range where histidine dissociates, especially at an alkaline pH.

SUMMARY

1. Sedimentation constants, diffusion coefficients and partial specific volumes of native ferro-, ferri-cytochrome c, and of ferri-cytochrome c modified by trichloroacetic acid were measured. Sedimentation constants at 20° in water ($S_{20,w}$) are 1.87, 1.91 and 2.50 Svedberg units for native ferro-, ferri- and modified ferri-cytochrome c, respectively. Diffusion coefficients at 20° in water ($D_{20,w}$) are 12.9×10^{-7}, 13.2×10^{-7} and 8.5×10^{-7} cm.^{2}/sec. for native ferro-, ferri- and modified ferri-cytochrome c, respectively. The partial specific volumes of these different kinds of cytochrome c's are 0.70.

2. From these values, the molecular weight of the native cytochrome c is calculated to be 12,000 and that of modified ferri-cytochrome c to be 24,000. This indicates that the molecules of modified cytochrome c exist as dimers of molecules similar to those of native cytochrome c.

The author would like to express his thanks to Prof. K. Okunuki and Dr. T. Horio for their valuable guidance, and is grateful to Prof. T. Isemura and Prof. K. Fukai of this university for helpful advice on the physicochemical measurements.

REFERENCES

(*1*) Margoliash, E., *Nature*, **170**, 1014 (1952)

(*2*) Margoliash, E., *Biochem. J.*, **56**, 529 (1954)

(*3*) Morrison, M., Estabrook, R. W., and Stotz, E. H., *J. Am. Chem. Soc.*, **76**, 6409 (1954)

(*4*) Paul, K. G., *Acta. Chem. Scand.*, **5**, 389 (1951)

(*5*) Stotz, E. H., Morrison, M., and Marinetti, G., "Enzymes: Units of Biological Structure and Function" Etd. Gaebler, O. H., Academic Press, N. Y., p. 401 (1956)

(*6*) Keilin, D., and Hartree, E. F., *Biochem. J.*, **39**, 289 (1945)

(*7*) Henderson, R. W., and Rawlinson, W. A., *Biochem. J.*, **62**, 21 (1956)

(*8*) Bodo, G., *Nature*, **176**, 829 (1955)

(*9*) Kuby, S. A., Palèus, S., Paul, K. G., and Theorell, H., *Acta Chem. Scand.*, **10**, 148 (1956)

(*10*) Hagihara, B., Morikawa, I., Sekuzu, I., Horio, T., and Okunuki, K., *Nature*, **178**, 630 (1956)

(*11*) Hagihara, B., Sekuzu, I., Tagawa, K., Yoneda, M., and Okunuki, K., *Nature*, **181**, 1588 (1958)

(*12*) Hagihara, B., Tagawa, K., Morikawa, I., Shin, M., and Okunuki, K., *Nature*, **181**, 1589 (1958)

(*13*) Horio, T., Higashi, T., Kusai, K., Nakai, M., and Okunuki, K., *Nature*, **182**, 1307 (1958)

(*14*) Hagihara, B., Horio, T., Nozaki, M., Sekuzu, I., Yamashita, J., and Okunuki, K., *Nature*, **178**, 632 (1956)

(*15*) Nozaki, M., Yamanaka, T., Horio, T., and Okunuki, K., *J. Biochem.*, **44**, 453 (1957)

(*16*) Nozaki, M., Mizushima, H., Horio, T., and Okunuki, K., *J. Biochem.*, **45**, 815 (1958)

(*17*) Yamanaka, T., Mizushima, H., Nozaki, M., Horio, T., and Okunuki, K., *J. Biochem.*, **46**, 121 (1959)

(*18*) Mizushima, H., Nozaki, M., Horio, T., and Okunuki, K., *J. Biochem.*, **45**, 845 (1958)

(*19*) Widmer, C., and Crane, F. L., *Biochim. et Biophys. Acta*, **27**, 203 (1958)

(*20*) Theorell, H., and Åkesson, A., *Acta Chem. Scand.*, **63**, 1818 (1941)

(*21*) Svedberg, T., and Pedersen, K. O., "The Ultracentrifuge", Oxford University Press, New York, (1940)

(*22*) Moriguchi, N., *J. Chem. Soc. Japan*, **53**, 377 (1932)

(*23*) Neurath, H., *Chem. Revs.*, **30**, 357 (1942)

(*24*) Pedersen, K. O., and Andersson, K. J. I., "The Proteins: Chemistry, Biological Activity, and Methods" Etd. Neurath, H., and Bailey, K., Academic Press, N. Y., Vol. I, p. 634, (1953)

(*25*) Isemura, T., and Fujita, S., *J. Biochem.*, **44**, 443 (1957)

(*26*) Polson, A. G., Thesis, University of Stellenbosch (1937)

(*27*) Theorell, H., *Biochem. Z.*, **285**, 207 (1936)

(*28*) Minakami, S., Titani, K., and Ishikura, H., *J. Biochem.*, **44**, 535 (1957)

(*29*) Tuppy, H., and Palèus, S., *Acta Chem. Scand.*, **9**, 353 (1955)

(*30*) Theorell, H., *Biochem. Z.*, **279**, 463 (1935)

(*31*) Hagihara, B., Morikawa, I., Sekuzu, I., and Okunuki, K., *J. Biochem.* **45**, 551 (1958)

(*32*) Hagihara, B., Yoneda, M., Tagawa, K., Morikawa, I., Shin, M., and Okunuki, K., *J. Biochem.*, **45**, 565 (1958)

(*33*) Hagihara, B., Tagawa, K., Morikawa, I., Shin, M., and Okunuki, K., *J. Biochem.*, **45**, 725 (1958)

(*34*) Hagihara, B., Tagawa, K., Sekuzu, I., Morikawa, I., and Okunuki, K., *J. Biochem.*, **46**, 11 (1959)

(*35*) Hagihara, B., Tagawa, K., Morikawa, I., Shin, M., and Okunuki, K., *J. Biochem.*, **46**, 321 (1959)

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MECHANISM OF CYANIDE RESISTANCE IN ACHROMOBACTER

II. ADAPTIVE FORMATION OF CYANIDE RESISTANT RESPIRATORY SYSTEM IN RESTING CELLS

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(Received for publication, September 29, 1959)

In the previous paper (*1*), it was reported that one strain of *Achromobacter* isolated from soil was found to give adaptive cyanide resistant growth and respiration, and that its cyanide resistant respiration was caused by the increase in the amount of cytochrome a_2 (or a_1), activity of which was strongly inhibited by cyanide.

During the course of studies on the mechanism of adaptation to cyanide in the bacteria, it was found that the cyanide resistant respiratory system could be formed adaptively even in resting cells. This paper reports studies on the adaptive formation of cyanide resistant terminal respiratory system in resting cells, and on the effect of chloramphenicol and streptomycin on the process of adaptation.

EXPERIMENTALS

Methods of cultivation of the organism, preparation of bacterial subcellular fractions, estimation of cyanide and estimation of cytochrome components, as well as chemical materials used were the same as those described in the previous report (*1*).

Casamino acid, a vitamin free acid hydrolyzed casein U. S. P., was used as amino acids mixture.

RESULTS

Adaptive Formation of Cyanide Resistant Respiratory System in Resting Cells—The cells grown in broth was collected and well washed with 0.5 per cent saline. When the washed cells were suspended in a medium containing $2\times10^{-2}\,M$ of glucose and $10^{-3}\,M$ of cyanide in $M/15$ phosphate buffer (pH 7.4) to make the cell concentration the same as that in growing medium and incubated aerobically at 30°, the cyanide resistant respiratory activity appeared gradually as shown in Fig. 1.

Effects of Nitrogen and Carbon Sources on Adaptation—Effects of amino acids, glucose and succinate on the adaptive formation of the cyanide resistant terminal respiratory system is shown in Table I. When the medium contained neither carbon nor nitrogen source, the adaptation could not occur even in the presence of cyanide. It was shown that the presence of glucose was

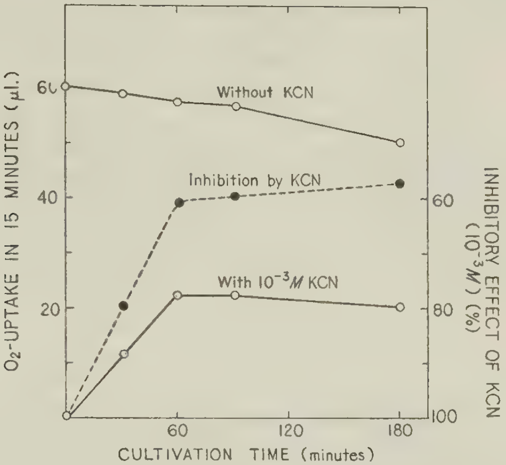

FIG. 1. Formation of cyanide resistant respiratory system in resting cells. Method of incubation; Each incubation tube contained 200 μM of glucose, 10 μM of KCN and 8×10^8 bacterial cells in 10 ml. of $M/15$ phosphate buffer (pH 7.4). The incubations were carried out aerobically at 30° on Monod's shake culture apparatus. After that, the cells were collected by centrifugation and subjected to the measurement of oxidative activity. Measurement of oxidative activity; Each Warburg vessel contained 0.2 ml. of 10^{-2} M KCN or H_2O, 3×10^8 incubated bacterial cells and 1.8 ml. of $M/15$ phosphate buffer (pH 7.4) in main compartment, 0.2 ml. of $M/5$ succinate in side arm and 0.2 ml. of 20% KOH in center well.. Reactions were carried out at 30°. Total volume was 2.2 ml..

TABLE I

Effect of Carbon and Nitrogen Sources on Adaptation

Composition of incubation medium			Inhibitory effect of cyanide (10^{-3} M) on succinate oxidation by intact cell
KCN (10^{-3} M)	Carbon source (2×10^{-2} M)	Casamino acid 0.2%	
+	–	–	98%
+	glucose	–	54
+	succinate	–	97
+	–	+	71
+	glucose	+	21

The incubation conditions and the method of the measurement of oxidase activity were similar to those given in Fig. 1. The incubation time was 90 minutes.

effective for the adaptation, whereas succinate was found to be ineffective. Since a small amount of amino acids showed a remarkable stimulative effect on the adaptation, it may be suggested that the protein synthesis occurred during the course of adaptation to cyanide.

Effect of Cyanide Concentrations on Adaptation—As shown in Table II, the optimal concentration of cyanide for the adaptation was found to be in the

TABLE II

Effect of Cyanide Concentrations on Adaptation

Concentration of cyanide in incubation medium	Inhibitory effect of cyanide (10^{-3} *M*) on succinate oxidation by resting cell
0 *M*	98%
10^{-4}	90
10^{-3}	34
2×10^{-3}	36
5×10^{-3}	76

Each incubation medium contained 200 μM of glucose, 20 mg. of casamino acid and KCN in 10 ml. of *M*/15 phosphate buffer (pH 7.4). Incubation time was 90 minutes. The method of the measurement of oxidative activity were similar to those given in Fig. 1.

TABLE III

Changes in Amount of Cells during Adaptation

Incubation time (hour)	Per cent increase in		
	Turbidity$^{1)}$	Dry weight$^{2)}$	Total nitrogen$^{3)}$
0	100	100	100
1	99	98	98
2	106	104	103
3	130	122	113

Each incubation tube contained 200 μM of glucose, 20 μM of KCN, 20 mg. of casamino acid and 8×10^{8} bacterial cells in 10 ml. of *M*/15 phosphate buffer (pH 7.4). The conditions are the same given in Fig. 1. 1) Turbidity was measured nepherometrically by Kotaki Model D Nepherometer. 2) After washing three times with water, bacterial cells were dried at 110°. 3) Bacterial cells were decomposed by H_2SO_4 using the mixture of K_2SO_4 and $CuSO_4\cdot5H_2O$ (1 : 3) and selenium as catalyser (*2*). Ammonium ion thus formed was distilled by steam followed by the colorimetric determination after Nesslerization.

range between 10^{-3} *M* and 2×10^{-3} *M*. In the case of 5×10^{-3} *M* of cyanide, though the adaptation occurred, the rate of the adaptation was very slow. In the following experiments, therefore, the adaptation was carried out in *M*/15 phosphate buffer (pH 7.4) containing 2×10^{-3} *M* cyanide, 2×10^{-2} *M* glucose and 0.2 per cent of casamino acid.

Change of Amount of Cells during the Course of Adaptation—The change of amount of cells during the course of adaptation was estimated by three ways as shown in Table III. No growth was detected during the initial two hours period, and only a slight growth was observed in succeeding one hour. This result strongly indicates that the adaptation occurred within two hours incubation is not accompanied by cellular growth.

Biochemical Basis for Change in Terminal Oxidase System during Course of Adaptation—The change of sensitivity to cyanide in the terminal oxidase system during the course of adaptation was also studied using a crude sonicate (as a succinoxidase system) and a subcellular particulate fraction (as a DPNH oxidase system). The methods for the preparation of the sonicate and the particulate fraction were described in previous paper (*1*). As shown in Fig. 2, the terminal oxidase system gradually become cyanide resistant along with the adaptation of the respiration of intact cells to cyanide. Therefore, the change in the amount of cytochromes in cells during the course of adaptation was observed spectrophotometrically. Fig. 3 shows the difference spectra of the particulate fractions obtained from adapted and non-adapted cells. As in the case of the adaptation in growing cells (*1*), the remarkable increase in the amount of cytochrome a_2 was observed in this

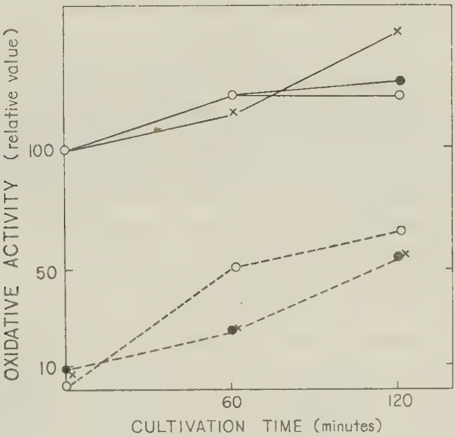

Fig. 2. Adaptation of terminal oxidase system to cyanide during the incubation. —○— Succinic oxidation activity of intact cells. —●— Succinoxidase activity of particulate fraction. —×— DPNH oxidase activity of crude sonicate. —— without cyanide, ---- with 10^{-3} *M* cyanide. The incubation condition is the same given in Table III, except that each incubation flask (500 ml.) contained 100 ml. of incubation medium. The method of the measurement of succinic oxidation activity of intact cells was the same given in Fig. 1. Both succinoxidase activity and DPNH oxidase activity of subcellular fractions were measured as described in previous paper (*1*).

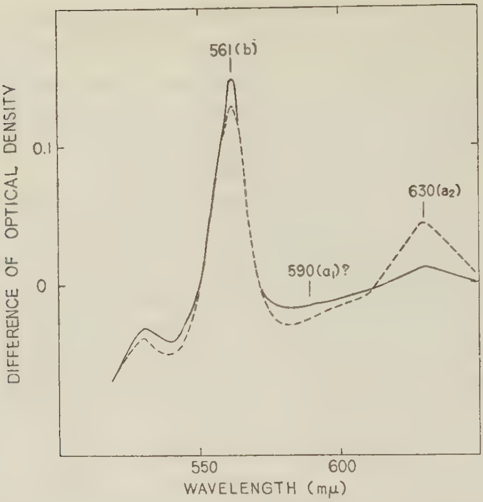

FIG. 3. Change of cytochrome spectrum during adaptation. The curves show the difference spectra (reduced with dithionite minus oxidized). —— Particulate fraction of non-adapted cells obtained from 2 liters culture. ---- Particulate fraction of adapted cells obtained from 2 liters culture. Protein concentrations were equal in both cases. Incubation for the adaptation was carried out for 90 minutes at 30°.

TABLE IV

Effect of Streptomycin and Chloramphenicol on Growth and Adaptation

Antibiotics	Concentration	(A) Inhibition of growth in broth	(B) Inhibition of adaptation to KCN in respiration
Streptomycin	10^{-3} *M*	95%	0%
	10^{-4}	40	0
Chloramphenicol	10^{-4}	95	100
	10^{-5}	30	95
	10^{-6}	5	22

In the experiments indicated in Column A, the bacteria were aerobically grown in cyanide free broth using Monod's shake culture apparatus at 30° in presence or absence of antibiotics.

In the experiments indicated in Column B, the cells grown in cyanide free broth were used as resting cells and tested their adaptability to cyanide. The method of the adaptation to cyanide was the same given in Table III and the method of the measurement of activity was the same given in Fig. 1.

Degree of inhibition of adaptation was defined as follow;

$$\left(1-\frac{Q_{O_2}\text{ in presence of cyanide }(10^{-3}\ M)\text{ of cells adapted to cyanide in presence of antibiotics}}{Q_{O_2}\text{ in presence of cyanide }(10^{-3}\ M)\text{ of cells adapted to cyanide in absence of antibiotics}}\right)\times 100$$

case. The absorption band of a_1 was so faint that no difinite conclusion about the change of its amount could be reached.

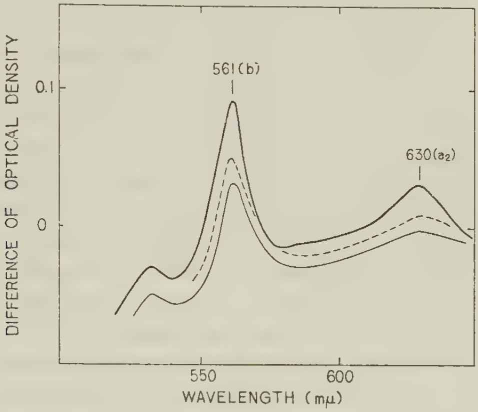

Fig. 4. Effect of chloramphenicol on change of cytochrome spectrum of particulate fraction during adaptation to cyanide. —— Incubated in the presence of 10^{-5} *M* chloramphenicol. —— Incubated in the absence of chloramphenicol. ---- Without incubation. The incubation conditions are the same given in Fig. 3.

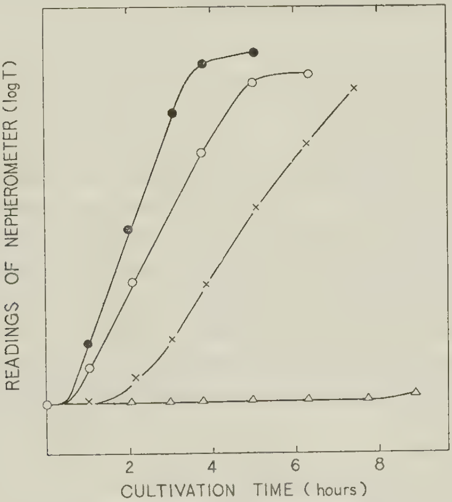

Fig. 5. Mode of action of chloramphenicol and cyanide on bacterial growth. The bacteria were grown in bouillon medium containing followings; —●— Nothing. —○— Chloramphenicol (10^{-5} *M*). —×— KCN (10^{-3} *M*). —△— Chloramphenicol (10^{-5} *M*) plus KCN (10^{-3} *M*).

The results described above were very similar to those of the adaptation in growing cells. Therefore, the adaptation to cyanide in resting cells, may be considered to proceed by the same mechanism as in growing cells.

Effect of Streptomycin and Chloramphenicol on Adaptation—The effects of streptomycin and chloramphenicol on bacterial growth and on adaptation to cyanide are shown in Table IV. Streptomycin (10^{-3} *M*) completely inhibited the growth, whereas it did not inhibit the adaptation. On the other hand, though chloramphenicol (10^{-5} *M*) inhibited the growth only partially, it inhibited the adaptation to cyanide almost completely. Chloramphenicol (10^{-5} *M*) was also found to inhibit the increase in the amount of cytochrome a_2 in resting cells during the incubation with cyanide, glucose and amino acids as shown in Fig. 4. Thus it was also comfirmed in resting cells that the increase in the amount of cytochrome a_2 (or a_1) is essentially involved in the mechanism of adaptation to cyanide. Also in this case, the observation could not be carried out on cytochrome a_1 owing to its minute amount. These results strongly suggest that the increase in the amount of cytochrome a_2 reflecting in the cyanide adaptation, seemed to be affected by the presence of chloramphenicol. The inhibitory effect of chloramphenicol on the adaptation to cyanide (not on the growth) was also manifested from the result shown in Fig. 5. The addition of both chloramphenicol and cyanide in the culture medium caused the complete inhibition of the bacterial growth, though each one of them could not inhibit the bacterial growth remarkably. In this case, it may be natural to consider that cyanide inhibits the growth of non-adapted cells and chloramphenicol inhibits the adaptation to cyanide, respectively.

DISCUSSION

The studies on the adaptation to a drug in resting cells is though to be important, as it is the first step to make clear the mechanism of adaptation to drugs in microorganisms. The results described above show that cyanide, energy source and amino acids are required for the adaptation to cyanide. This fact indicates that the synthesis of protein occurred during the adaptation. The cytochrome a_2 may be one of the protein of this kind.

Another interesting fact is that glucose could not be replaced by succinate as an energy source during the adaptation. The followings are the possible explanations of this phenomenon. a) In connection with the protein synthesis, the nucleic acid synthesis may also occur during the adaptation. Ribose moiety of nucleic acid must, therefore, be formed in this step. As the precursor of ribose, glucose seems to be much better than succinate, considering the position of these compounds on the metabolism. b) As shown in the previous paper (*1*), though the respiration of non-adapted cells was almost completely inhibited by cyanide, its fermentation may be resistant to 10^{-3} *M* of cyanide. Therefore, when glucose is used as an energy source, the energy required for the adaptation at the early stage may be produced by the fermentation, though succinate could not produce energy anaerobically. However, the precise study on this point is now under way.

As shown in Table IV, chloramphenicol (10^{-5} *M*) inhibited the adaptation to cyanide, but could not inhibit the cell growth in broth. During the course of the adaptation, chloramphenicol inhibited the increase in the amount of cytochrome a_2 which was thought to be essential in the adaptation. On the other hand, the fact that the equal concentration of chloramphenicol did not inhibit the normal growth of the bacteria indicates that it did not inhibit the formation of cytochrome a_2 during the growth in the absence of cyanide. In other words, chloramphenicol ($10^{-5}\,M$) does not inhibit the normal formation of cytochrome a_2 but inhibits the adaptive formation of cytochrome a_2. One of the possible difference between these two cases of the cytochrome formation seems to exist in the difference of their synthetic mechanisms. Another possibility is that there may be some factors which have a close relation with "adaptation", namely "to become resistant", though the mechanism of the formation of cytochrome a_2 may be the same in both cases. In any cases, there must be some difference between the mode of formation of cytochrome a_2 which exists in the normal cells and that of cytochrome a_2 which appears during the adaptation to cyanide.

SUMMARY

1. It was demonstrated that the respiratory system of a strain of *Achromobacter* could adaptively become cyanide resistant without bacterial growth.

2. During the adaptation, the amount of cytochrome a_2 in resting cells remarkably increased as in the case of the adaptation in growing bacteria.

3. Cyanide, energy source and amino acids were required for the adaptation. The optimal concentration of cyanide in the medium was $1\sim2\times10^{-3}\,M$.

4. Streptomycin ($10^{-3}\,M$) inhibited the bacterial growth but did not inhibit the adaptation to cyanide.

5. Chloramphenicol ($10^{-5}\,M$) only inhibited the adaptation to cyanide but did not inhibited the bacterial growth in bouillon medium.

6. The mechanism of the adaptation was discussed especially with regard to the mode of action of chloramphenicol.

The authors wish to express their sincere appreciation to Dr. K. Sakaguchi for his kind guidance throughout the course of this study.

REFERENCES

(*1*) Mizushima, S., and Arima, K., *J. Biochem.*, **47**, 351 (1960)
(*2*) Ma, T., S., and Zuazaga, G., *Ind. Eng. Chem. Anal. Ed.*, **14**, 280 (1942)

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STUDIES ON RIBONUCLEASES IN TAKADIASTASE

IV. ACTION OF RIBONUCLEASE T_1 ON DEAMINO-RIBONUCLEIC ACID

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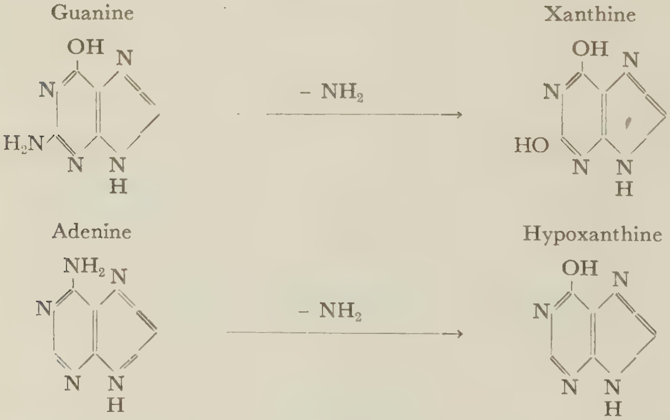
(Received for publication, September 30, 1959)

In the previous papers it was shown that RNase T_1** specifically hydrolysed the secondary phosphate ester bond of guanosine-3′ phosphate in RNA *via* guanosine-2′,3′ cyclic phosphate (*1*∼*3*). RNase T_1 cannot split the secondary phosphate ester bond of adenosine-3′ phosphate. It is probable that the specificity of the RNase T_1 depends upon the group attached to the 6 carbon atom of purine ring. If RNA is deaminated, the amino groups linked to 2 carbon atom of guanine and 6 carbon atom of adenine alter to hydroxyl (or keto) groups and these bases change to xanthine and hypoxanthine respectively. So in order to find out the base specificity of RNase T_1, digestion of deamino-RNA was pursued.

Scheme I



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** Following abbreviations are used : RNA : ribonucleic acid, deamino-RNA : deamino-ribonucleic acid, RNase T_1 : ribonuclease T_1, 3′-Gp : guanosine-3′ phosphate, $G_{\text{cyclic P}}$: guanosine-2′,3′ cyclic phosphate, 3′-Ip : inosine-3′ phosphate, $I_{\text{cyclic P}}$: inosine-2′,3′ cyclic phosphate, 3′-Xp : xanthosine-3′ phosphate, $X_{\text{cyclic P}}$: xanthosine-2′,3′ cyclic phosphate, PMase : phosphomonoesterase, Tris : tris(hydroxymethyl) aminomethane.

EXPERIMENTALS

Materials

RNA—A commercial yeast RNA (Schwarz Laboratories) was deproteinized by chloroform gel formation, dialyzed in a cellophane tube against distilled water in a cold room and precipitated with alcohol.

Deamino-RNA—Yeast RNA treated as above was deaminated by the method of Takemura (*4*): The mixture of yeast RNA solution (1 g./2 ml. water) and sodium nitrite solution (2.5 g./4.5 ml. water) was set under stirring in an ice bath for 5 hours with dropwise addition of 6.5 ml. of glacial acetic acid. The obtained deamino-RNA was purified by alcohol precipitation and dried with ether.

RNase T_1—The purest sample extracted and prepared from Takadiastase according to the method of Sato and Egami (*1*) was used.

Phosphomonoesterase (*PMase*)—PMase was prepared from human prostate by the method of Schmidt *et al.* (*5*), and 40 units of the enzyme were used for each experiment.

Methods

Organic phosphorus was measured by the method of Allen (*6*). Nitrogen was measured by micro-Kjeldahl method.

Mean Chain Length of Polynucleotide—Terminal phosphate was splitted by PMase and mean chain length was calculated by the ratio of total phosphate to inorganic phosphate produced from terminal phosphate.

Paper Chromatography—Tôyô roshi No. 51 paper was used throughout the study and spots on the chromatogram were located by usual photographic method. *Solvent* I (*7*). 70 ml. of tertiary butanol, 13.2 ml. of constant boiling hydrochloric acid and water to 100 ml.. *Solvent* II (*8*). Saturated ammonium sulfate : water : isopropanol (79 : 19 : 2). *Solvent* III (*9*). Isopropanol : water (7 : 3), with concentrated ammonia added to the bottom of the chromatographic vessel (0.5 ml. for each liter of gas space).

Degree of Hydrolysis—To find out the degree of hydrolysis uranium reagent soluble phosphate and inorganic phosphate which were splitted by PMase after hydrolysis of cyclic ester bonds in 0.1 *N* hydrochloric acid for 20 hours at 2° were measured.

Determination of Terminal Nucleotide—The terminal nucleotide of oligonucleotide produced by RNase T_1 digestion was determined as follows. The reaction mixtures containing RNA or deamino-RNA and RNase T_1 in Tris buffer were incubated for various time intervals at 37° and then in order to split the cyclic phosphate and to inactivate RNase T_1 the reaction mixtures were placed in 0.1 *N* hydrochloric acid for 20 hours at 2°. After neutralization PMase was added to them and they were incubated for 2 hours at 37°. Then the reaction mixtures were hydrolyzed in 1 *N* sodium hydroxide at 37° for 18 hours, and the nucleotides and nucleosides obtained were separated by paper chromatography. Each spot on the chromatogram was eluted with 0.1 *N* hydrochloric acid and its absorption spectrum was measured.

RESULTS

Properties of Deamino-RNA

Analysis of Phosphorus and Nitrogen Contents of Deamino-RNA—Yeast RNA and purified deamino-RNA were analyzed and the results are shown in Table I.

TABLE I
Phosphorus and Nitrogen Contents of RNA and Deamino-RNA

	P	N
RNA	8.55 %	14.57 %
Deamino-RNA	8.20 %	11.29 %

Absorption Spectrum—Ultraviolet absorption spectra of RNA and deamino-RNA were measured and their extinction coefficient were determined by measuring the phosphate.

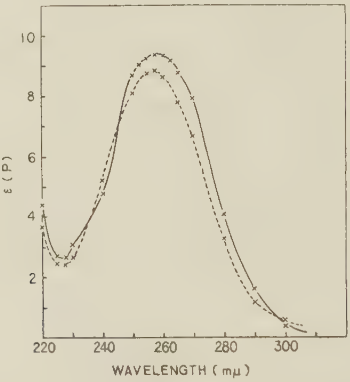


FIG. 1. Absorption spectra of yeast RNA and deamino-RNA. —— RNA, ---- Deamino-RNA.

Mean Chain Lengths of Yeast RNA and Deamino-RNA—Chain length was number of nucleotides units contained in a polynucleotide chain calculated by the terminal phosphate.

TABLE II
Chain Lengths of Yeast RNA and Deamino-RNA

Experimental No.	1	2	3	Mean
RNA	18.0	18.9	17.0	18
Deamino-RNA	7.8	7.6	7.7	8

Base Analysis—Base analysis of deamino-RNA was carried out using two methods. Deamino-RNA was hydrolyzed with 1 *N* HCl at 100° for 1 hour and purine bases and pyrimidine nucleotide were separated by paper chromatography using solvent I (*7*). In this experiment separation of purine bases was incomplete but at least it was shown that the deamino-RNA consisted of uracil, xanthine, hypoxanthine and trace amounts (less than 3 per cent) of guanine, adenine and cytosine. The results was confirmed by the other experiment; deamino-RNA was hydrolyzed with perchloric acid and the purine and pyrimidine bases produced were separated on the ion exchanger column (Dowex-50) (*10*).

Action of RNase T_1 on Deamino-RNA

Yeast RNA (10 mg.) or deamino-RNA (10 mg.) in 0.5 ml. Tris buffer (pH 7.5) were hydrolyzed by 1 μg. RNase T_1 at 37° for 0.2 and 10 hours.

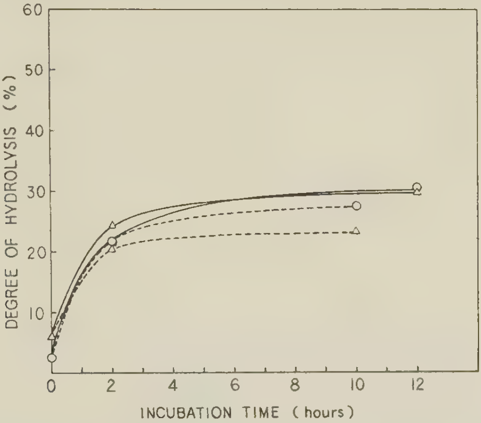


FIG. 2. Hydrolysis of yeast RNA by RNase T_1. ○: uranium reagent soluble phosphate, △: inorganic phosphate ——: RNase T_1 100 μg., ----: RNase T_1 1 μg. Condition of hydrolysis: RNA 10 mg., RNase T_1, EDTA 2×10^{-3} *M*, total 0.5 ml, Tris buffer pH 7.5, 0.1 *M*.

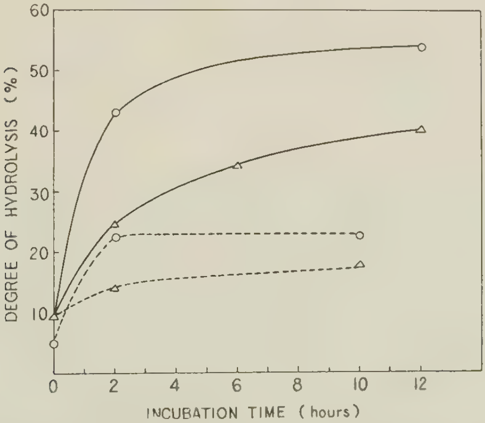


FIG. 3. Hydrolysis of deamino-RNA by RNase T_1. ○: uranium reagent soluble phosphate, △: inorganic phosphate, ——: RNase T_1 100 μg., ----: RNase T_1 1 μg. Condition of reaction was the same as in the case of Fig. 2.

Using aliquots of the reaction mixtures uranium reagent solublep hosphate and inorganic phosphate splitted by PMase were measured. Fig. 2 and Fig. 3 show the degree of hydrolysis of yeast RNA and deamino-RNA by RAase T_1

As shown in Fig. 2 and Fig. 3. deamino-RNA was hydrolyzed as well as yeast RNA. To identify the reaction products, the reaction mixtures were subjected to paper chromatography. (Fig. 4 I). Spot 2 and 3 were eluted with distilled water. The absorption curves of spot 2 and spot 3 were both the same as that of inosine. However, rechromatography of spot 2 after treatment with 0.1 *N* HCl for 20 hours at 2° gave a spot with R_f value identical with that of spot 3. Considering the result and specificity of RNase T_1 against yeast RNA, spot 2 seems to be that of $I_{\text{cyclic P}}$ and spot 3 to be that of 3′-Ip.

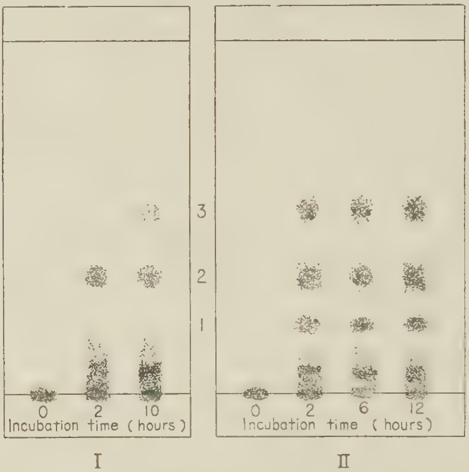


Fig. 4. Paper chromatograms of the reaction products of deamino-RNA digestion by RNase T_1. I: RNase T_1 1 μg. II: RNase T_1 100 μg. Condition of reaction was the same as in the case of Fig. 2. Solvent II was used. The spots are; (1) $X_{\text{cyclic P}}$, (2) $I_{\text{cyclic P}}$, (3) 3′-Ip.

To find out the bond splitted by RNase T_1 in deamino-RNA the determination of terminal nucleotide was carried out. After RNase T_1 digestion followed by acid treatment, PMase digestion and alkaline hydrolysis, reaction mixtures were neutralized and subjected to paper chromatography.

On the chromatograms shown in Fig. 5 I spot 2, 3 and 4 correspond to those of xanthosine, inosine and pyrimidine nucleoside, respectively. As shown in the Fig. 5 I clear spot of nucleoside was only that of inosine. Their amounts are measured by the U.V. absorption after elution with 0.1 *N* HCl (Fig. 6).

This results showed that RNase T_1 can split the secondary phosphate ester bonds of inosine-3′ phosphate in the condition described above.

Next, the same experiment was repeated but using 100 μg. RNase T_1 in 0.5 ml. of reaction mixture (100 times of that of the first experiment). Uranium reagent soluble phosphate and inorganic phosphate produced were shown in Fig. 2 and Fig. 3 together with the results of the first experiment. As shown in Fig. 2 RNA was digested by 1 μg. RNase T_1 as much as by 100 μg. RNase T_1, while as shown in Fig. 3 in deamino-RNA digestion considerable difference between by 1 μg. RNase T_1 and 100 μg. RNase T_1 was found. It was expected that in deamino-RNA digestion by 100 μg. RNase T_1 additional

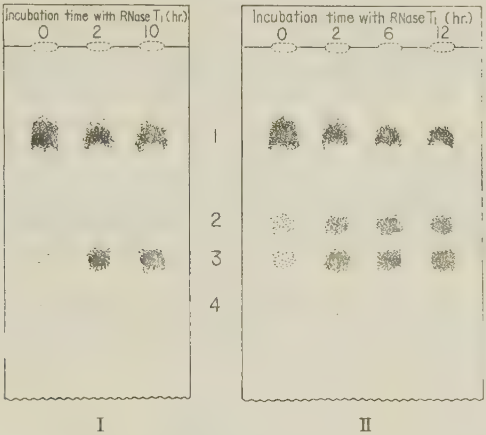

Incubation time with RNase T₁ (hr.)
0
2
10
Incubation time with RNase T₁ (hr.)
0
2
6
12
1
2
3
4
I
II

FIG. 5. Paper chromatograms of nucleotides and nucleosides coming from terminals in hydrolysis of deamino-RNA by RNase T_1 followed by acid treatment, PMase digestion and alkaline hydrolysis. Initial reaction mixtures were the same as in the case of Fig. 2. Solvent III was used. The spots are, (1) nucleotides, (2) xanthosine, (3) inosine, (4) uridine.

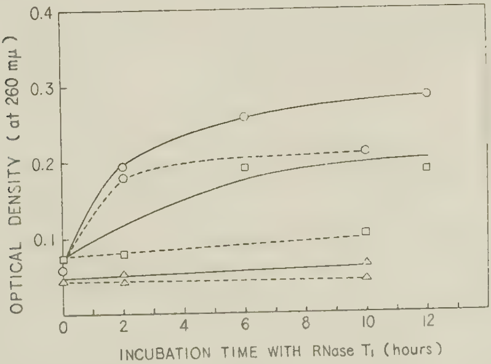


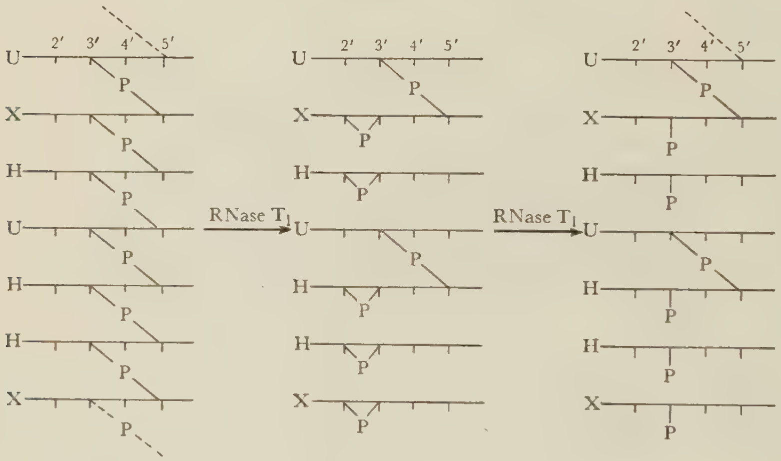
FIG. 6. Terminal nucleosides in hydrolysis of deamino-RNA by RNase T_1. ○: inosine, □: xanthosine, △: uridine, ——: RNase T_1 100 μg. ----: RNase T_1 1 μg.

bonds were splitted. So the reaction products were subjected to paper chromatography (Fig. 4 II). A new spot which could not be seen in 1 μg. RNase T_1 digestion appeared. The new spot was identified as that of xanthosine-2′,3′ cyclic phosphate and in the spot 2 in addition to $I_{\text{cyclic P}}$ slight amount of 3′-Xp was detected. It seems that in deamino-RNA digestion by RNase T_1 the secondary phosphate ester bond of xanthosine-3′ phosphate was hydrolyzed, as proved by terminal nucleotide investigation. On the paper chromatogram of terminal nucleoside shown in Fig. 5 II spot of xanthosine was found besides that of inosine. The spot was eluted and the increase of absorption maximum at every reaction time was pursued (Fig. 6). As shown in the figure xanthosine appeared and increased with the reaction time, while uridine did not change throughout the reaction.

DISCUSSION

It was clarified by the results described above that the RNase T_1 hydrolyzed the secondary phosphate ester bonds of 3′-Gp, 3′-Ip, and 3′-Xp with intermediary formation of respective cyclic nucleotides (Scheme II). But it could not hydrolyzed the secondary phosphate ester bond of 3′-Ap in RNA digestion. Therefore hydroxyl (or keto) group attached to the 6 carbon atom of purine ring seems to be a requirement for the action of RNase T_1. However with low concentration of the enzyme, phosphodiester bonds of xanthylic acid was scarcely hydrolyzed. It seems that substituent group attached to carbon atom 2 of purine ring itself interferes with the action of RNase T_1 or changes the electronic state of purine ring and decreases the affinity for the enzyme. It is very interesting that the specificity of RNase T_1 is influenced by substituent of purine rings.

SCHEME II



—┬—┬—┬—┬— : ribose, P : Phosphate, U : Uracil, X : Xanthine,
H : Hypoxanthine

Moreover it should be noted that two new substances, inosine-2′,3′ cyclic phosphate and xanthosine-2′, 3′ cyclic phosphate were obtained by deamino-RNA digestion by RNase T_1 (*11*). These nucleotides may be useful as substrates for the research of specificity of ribonucleases in general, and may be also interesting materials as substrates for synthetic reaction by RNase T_1 (*12*).

SUMMARY

1. It was proved that RNase T_1 could digest the deamino-yeast RNA and hydrolyze the secondary phosphate ester bonds of inosine-3′ phosphate and xanthosine-3′ phosphate with the intermediary formation of respective nucleoside-2′,3′ cyclic phosphate during the digestion.

2. It seems that for the action of RNase T_1 hydroxyl (or keto) group attached to the carbon atom 6 of purine ring is necessary.

3. Two new compounds, inosine-2′,3′ cyclic phosphate and xanthosine-2′,3′ cyclic phosphate, were obtained from the digestion mixture of deamino-yeast RNA by RNase T_1.

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REFERENCES

(*1*) Sato, K., and Egami, F., *J. Biochem.*, **44**, 753 (1957)
(*2*) Sato, K., and Egami, F., *Compt. rend. soc. biol.*, **151**, 1792 (1957)
(*3*) Sato-Asano, K., *J. Biochem.*, **46**, 31 (1959)
(*4*) Takemura, S., *J. Chem. Soc. Japan.*, (*Nippon Kagaku Zasshi*), **72**, 674 (1951)
(*5*) Schmidt, G., Cubiles, R., Zöllner, N., Hecht, L., Strickler, N., Seraidarian, K., Seraidarian, M., and Thannhauser, S. J., *J. Biol. Chem.*, **192**, 715 (1951)
(*6*) Allen, R. J., *Biochem. J.*, **34**, 858 (1940)
(*7*) Smith, J. D., and Markham, R., *Biochem. J.*, **46**, 509 (1950)
(*8*) Markham, R., and Smith, J. D., *Biochem. J.*, **49**, 401 (1951)
(*9*) Markham, R., and Smith, J. D., *Biochem. J.*, **52**, 558 (1952)
(*10*) Wall, J. S., *Anal. Chem.*, **25**, 950 (1953)
(*11*) Sato-Asano, K., Fujii, Y., and Egami, F., *Bull. Chem. Soc. Japan*, **32**, 1068 (1959)
(*12*) Sato-Asano, K., and Egami, F., *Biochim. et Biophys. Acta*, **29**, 606 (1958) Sato-Asano, K., *J. Biochem.*, in press

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STUDIES ON THE ADENOSINE TRIPHOSPHATE-PHOSPHATE EXCHANGE REACTION

II. THE STATE OF ELECTRON CARRIERS AND THE ADENOSINE TRIPHOSPHATE-PHOSPHATE EXCHANGE REACTION

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In the preceding paper (*1*) it was shown that the ATP-*i*P exchange reaction proceeds during oxidative phosphorylation, even with ADP* as an acceptor of *i*P and a hexokinase-glucose system as trapper of the ATP formed by adenylate kinase. Moreover there was less exchange during oxidative phosphorylation with succinate as substrate than in a system which did not involve electron transfer.

The exchange reaction was studied further to see why the passage of electrons in relation to oxidative phosphorylation inhibited the reaction.

This paper shows that the inhibition of the exchange during oxidation of succinate is probably due to the reduced state of cytochrome b (and related enzyme at the same level). Slater *et al.* (*2*, *3* and *4*), found three pH optima for the activity of DNP-activated ATP-ase and for the P/O ratio. These were also found for the exchange reaction and seem to be related to the oxidation-reduction state of the electron carriers.

MATERIALS AND METHODS

The preparation of mitochondria from rat liver and ATP, and the determinations of *i*P^{32} incorporated into organic materials, oxygen consumption, *i*P and radioactivity were made as previously reported (*1*).

Cytochrome c was prepared in crystalline form from bovine heart muscle as previously reported (*5*).

The pH was measured with a glass electrode.

RESULTS

Effect of Cyanide on the Exchange Reaction —As reported previously (*1*), under conditions where was not only oxidative phosphorylation with succinate as substrate, but also the ATP-*i*P exchange reaction, various concentrations of

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* The following abbreviations are used in this paper: ATP, adenosine triphosphate; ADP, adenosine diphosphate; *i*P, inorganic orthophosphate; DNP, 2,4-dinitrophenol; DPN, diphosphopyridine nucleotide.

KCN were added to the reaction mixture to the reaction mixture to reduce the electron carriers. Thus the effect of the state of the carriers on the exchange activity was investigated. Table I shows the exchange activity as calculated by subtracting the decrease in *i*P from the incorporation of *i*P^{32} into organic phosphate. As already observed (*1*) this was reduced in the presence of succinate. A low concentration of KCN ($10^{-4} \sim 10^{-3}$ *M*) with the succinate stimulated the reaction in proportion to the inhibition of oxygen consumption. At a high concentration of KCN (10^{-2} *M*) the reaction was inhibited. When the mitochondria were preincubated with succinate and the reaction started by addition of ATP or *i*P^{32}, the effect of the concentration of KCN on the exchange reaction was shown more clearly.

TABLE I

Effect of Cyanide on Oxidative Phosphorylation and the ATP-iP Exchange Reaction

The contents of the manometer flask were as follows: Main chamber *i*P^{32}, 10.1 (Exp. 1) or 13.4 (Exp. 2) μ moles; ATP, 6 μ moles; KF, 5 μ moles; EDTA, 1 μ mole; glucose, 20 μ moles; hexokinase, 1 mg. of preparation (amount which reacted with 2 μ moles of ATP under these conditions); water to 0.9 ml. Side arm 0.1 ml. of mitochondrial suspension in 0.3 *M* sucrose. Centre well 0.1 ml. of 20% KOH. pH 7.2. Temperature 25°. Reaction period, 20 minutes. In Exp. 1 the reaction were started by addition of the mitochondrial suspension and in Exp. 2 by addition of ATP-*i*P. Radioactivity of *i*P^{32} is about 10^4 c.p.m. per μ moles.

Exp.	Succinate	KCN (final concentration	O_2 consumption (μ atoms)	*i*P^{32} incorporated into organic compound (μ moles) (a)	*i*P decrease (μ moles) (b)	Exchange activity (a)-(b)
1	−	−	0.36	1.51	0.0	1.51
	+	−	2.64	2.62	2.01	0.61
	+	10^{-4} *M*	2.15	2.75	1.62	1.13
	+	10^{-3} *M*	0.12	0.81	0.0	0.81
	+	2×10^{-3} *M*	0.0	0.66	—	0.66
	+	10^{-2} *M*	0.0	0.36	—	0.36
2	−	−	0.56	1.83	0.0	1.83
	+	−	4.18	3.11	2.84	0.27
	+	10^{-4} *M*	2.87	3.36	2.10	1.26
	+	10^{-3} *M*	0.33	1.44	0.0	1.44
	+	2×10^{-3} *M*	0.0	0.63	—	0.63
	+	10^{-2} *M*	0.0	0.34	—	0.34

Slight reduction of the carriers by inhibition of the oxidase activity seems to stimulate the exchange reaction slightly.

Effect of Antimycin A—The ATP-*i*P exchange reaction was slightly affected by addition antimycin A without shaking the reaction. It was much

TABLE II

Effect of Antimycin A on the ATP-iP Exchange Reaction

Each reaction mixture contains in a total volume of 1 ml. $MgCl_2$, 5 μ moles; KF, 10 μ moles; EDTA, 4 μ moles; *i*P (10^4 c.p.m. per μ mole of *i*P), 7 μ moles and 0.1 ml. of mitochondrial suspension in 0.3 *M* sucrose. pH, 7.2. Temperature, 25°. Reactions were started by addition of ATP and *i*P. Reaction period, 10 minutes without shaking.

Succinate added (20 μ moles)	Antimycin A added (μg.)	Exchange activity (μ moles)
–	–	1.15
+	–	1.36
–	0.5	1.14
+	0.5	0.70
–	1.0	1.04
+	1.0	0.57
–	2.0	0.78
+	2.0	0.44

TABLE III

ATP-iP Exchange Activity and Condition of Electron Carriers at Oxidase Level

Experimental conditions: (A) as in Table I, Exp. 2 with the exception that no glucose-hexokinase was added. (B) as in Table II in Thunberg tubes. Materials added were as follows: Cytochrome c, 0.4 μ moles; ascorbate, 20 μ moles; antimycin A, 1 μg.

(A) With shaking in Warburg apparatus. (Reaction period, 10 minutes)

Exp. No.	Gas phase	Additions	Exchange activity (μ moles)	O_2 consumption (μ atoms)
1	Air	None	0.95	0.0
		Cytochrome c	1.29	0.0
		,, + ascorbate	1.11	1.00
		,, + antimycin A	1.21	0.14
		Antimycin A	0.96	0.0

(B) Without shaking in Thunberg tubes. (Reaction period, 10 minutes)

Exp. No.	Gas phase	Additions	Exchange activity (μ moles)
2	Air	None	1.51
	Nitrogen	Cytochrome c	1.02
	Nitrogen	,, + ascorbate	0.96
	Air	,, + ascorbate	1.74
	Air	Antimycin A	1.58
	Nitrogen	,, + cytochrome c	0.83
	Nitrogen	,, + ,, + ascorbate	0.65
	Air	,, + ,, + ascorbate	1.36

inhibited by further addition of succinate (Table II).

It is known that antimycin A inhibits the electron transfer system by blocking of electron transport between cytochrome b and c_1. Cytochrome b is probably strongly reduced by addition of succinate. Therefore reduction of cytochrome b probably suppresses the exchange activity. This conclusion is supported by the results of Wadkins and Lehninger *(6)* which show a decrease in the exchange activity on reduction of the electron carriers.

Effect of Oxidation-Reduction State of the Electron Carriers at the Oxidase Level—If the exchange reaction is a reversal of oxidative phosphorylation, there might also be inhibition by reduction of carriers at the oxidase level as well as by cytochrome b.

Under aerobic conditions this was studied using a Warburg apparatus and under anaerobic condition by using a Thunberg tubes. Ascorbate, antimycin A, and cytochrome c were added to the reaction mixtures as shown in Table III. Although, for some reason, cytochrome c stimulated the exchange, antimycin A and ascorbate were without affect. Completely anaerobic conditions were obtained by evacuation of the air. Uuder these conditions activity was much less than under aerobic conditions and without

TABLE IV

ATP-iP Exchange Reaction with Various Gas Phase

The column of gas phase "Nitrogen → Air" indicates that manometric vessels filled with nitrogen gas were shaken for 5 minutes at 25°, and were then refilled with air before the reaction was started. 20 μ moles succinate were added. Other experimental conditions as in Table III (A).

Exp. No.	Gas phase	Additions	Exchange activity (μ moles)	iP decrease (μ moles)	O_2 consumption (μ atoms)
1	Air	None	0.76	1.58	0.51
		Succinate	0.46		2.30
		,, + antimycin A	0.44		0.19
	Nitrogen → air	None	1.13	1.05	0.36
		Succinate	0.75		2.31
		,, + antimycin A	0.50		0.26
	Nitrogen	None	0.98		—
		Succinate	0.68		—
		,, + antimycin A	0.54		—
2	Air	None	1.86		—
	Air	Antimycin A	1.81		—
	Air	,, + succinate	1.48		—
	CO (90%)+air	,, + succinate	1.46		—
	Air	,, + succinate + ascorbate	1.46		—

shaking. With antimycin A and succinate, the exchange was not affected by replacing the air by a mixture of 90 per cent carbon monoxide and 10 per cent air or by the addition of ascorbate (Table IV, Exp. 2). This result is similar to those of the previous report (*1*) in which there was no effect of ferrocyanide, ascorbate or ferricyanide. From these results apparently, the oxidation-reduction state of carriers at the oxidase level has no effect on the reaction. This conclusion is not supported by the results described below.

Effect of the Gas Phase on the Exchange To study the effect of changes of the oxidation-reduction state of the carriers on the ATP-*i*P exchange reaction, the reaction mixtures were preincubated in Warburg vessels under air or nitrogen. Before incubation the nitrogen was replaced by air in some vessels, Table IV.

Activity was highest when reactions were preincubated under nitrogen and incubated under air. It was moderate when preincubated and incubated under nitrogen. Activity was lowest when preincubated and incubated under air. These results were the same for the three reaction systems: no addition, addition of succinate, and addition of succinate and antimycin A. When preincubated and incubated under nitrogen, which presumably gave the

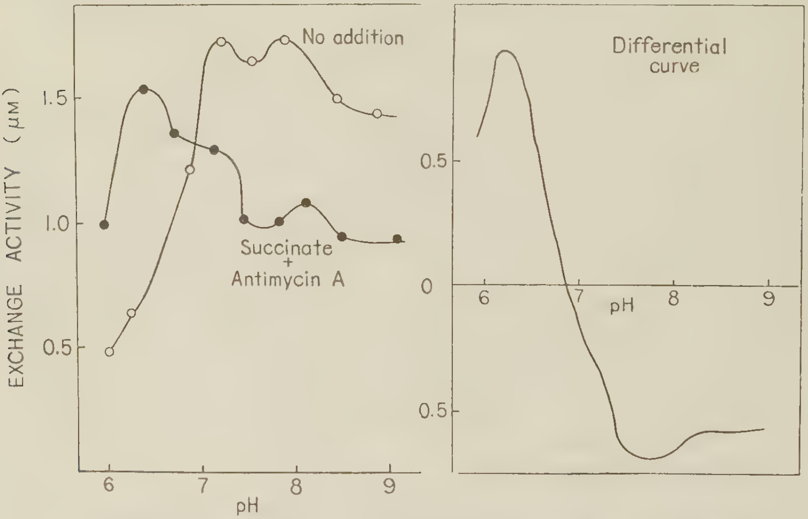

Fig. 1. Effect of pH on the ATP-*i*P exchange and the effect of the oxidation-reduction state of the electron carriers. (I) Effect of addition of succinate and antimycin A.

Reaction mixture contained in 1 ml. of total volume, EDTA, 1 μmole; MgCl$_2$, 5 μmoles; KF, 10 μmoles; ATP, 5 μmoles; iP^{32} (ca. 2×10^4 c.p.m. per μmole of *i*P) 7 μmoles. 20 μmoles and 1 μg. of succinate and antimycin A were added respectively. Reaction were started by addition of 0.1 ml. of mitochondrial suspension in 0.3 *M* sucrose. Temperature, 25°. Reaction period, 10 minutes. Left, estimated curves. Right, differential curve.

most reduced state of the three conditions, the exchange reaction was more than when air was used for both preincubation and incubation and the reaction mixture was kept in the oxidized state.

Three pH Optima in the ATP-iP Exchange Reaction—Recently Myers and Slater (*2*, *3*) found three pH optima, *i.e.* pH 6.3, 7.4 and 8.5, with DNP-activated ATP-ase. These may be related to the three steps in respiratory chain which are coupled with phosphorylation. Furthermore Hulsmann and Slater (*4*) reported that there are three pH optima in the P/O ratio of mitochondria. They state that the three pH optima of oxidative phosphorylation correspond to specific oxidative phosphorylation steps: the optimum at pH 6.3 to cytochrome oxidase, at pH 7.4 to cytochrome b (in the middle of respiratory chain) and at pH 8.5 to oxidizing enzymes of reduced DPN.

These three peaks were also observed in the exchange reaction (Fig. 1-3). Fig. 1 shows the activity in relation to the pH of the medium without added oxidizable substrate. There were two optima at pH 7.2 and 7.9. With added succinate and antimycin A there were three optima at pH 6.3, 7.2

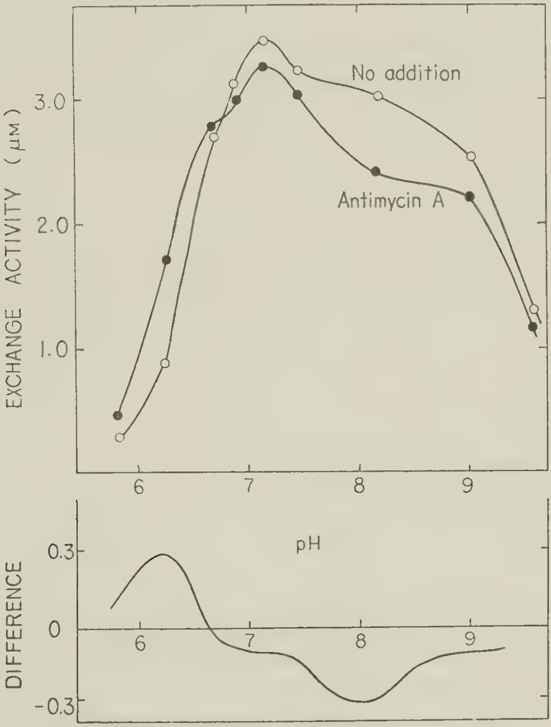

FIG. 2. Effect of pH on the ATP-*i*P exchange and the effect of the oxidation-reduction state of the electron carriers. (II) Effect of addition of antimycin A.

Other experimental conditions as in Fig. 1.

and 8.1. When the activity of the control was subtracted from that of the reaction containing succinate and antimycin A, a distinct peak was found at pH 6.3, while the small peak at pH 7.2 and the large peak at pH 7.8 decreased. Probably on addition of succinate and antimycin A, the oxidase level carrier was oxidized while cytochrome b and the DPN level carrier were reduced. The optimum at pH 6.3 may correspond to oxidase level phosphorylation, the peak at pH 7.2 to cytochrome b level phosphorylation and the pH at 7.8 to DPN level phosphorylation, though the pHs disagreed with those of Slater's results. If so it could be concluded that the exchange reaction was stimulated by the oxidized state and inhibited by the reduced state of the carriers.

The peaks in the activity curve are not caused by the action of antimycin A on the exchange reaction, as seen in Fig. 2. When the exchange reaction proceeds in the absence of substrate, the curve obtained by the difference between reactions in the presence and absence of antimycin A show peaks of similar height to those in Fig. 1.

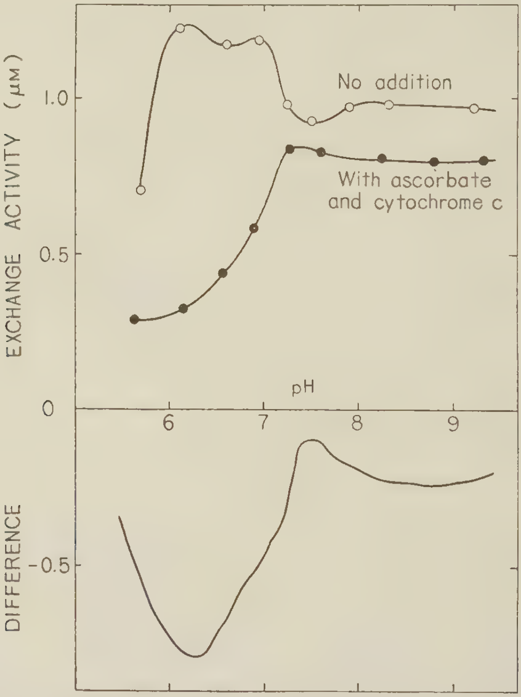

Fig. 3. Effect of pH on the ATP-^{i}P exchange and the effect of the oxidation-reduction state of the electron carriers. (III) Effect of addition of ascorbate and cytochrome c under anaerobic conditions.

20 μmoles and 0.2 μmoles of ascorbate and cytochrome c were added respectively. Anaerobic conditions were obtained in a Thunberg tube under nitrogen. Other experimental conditions as in Fig. 1.

Incubations were made under extremely anaerobic conditions in Thunberg tubes, replacing the air by nitrogen. The effect of addition of ascorbate with cytochrome c on the exchange reaction was examined. Results are shown in Fig. 3.

In the presence of ascorbate the two distinct peaks at pH 6.3 and 8.3 disappeared. The exchange reaction at pH 6.3 was probably strongly inhibited by reduction of the oxidase level carrier. Exchange at pH 8.5 was only slightly inhibited by reduction of the DPN level carrier, while exchange at pH 7.2 was unchanged because the same oxidation-reduction state existed as under anaerobic conditions in the absence of ascorbate-cytochrome c.

DISCUSSION

Some work has been done on the relation of the ATP-*i*P exchange reaction to oxidative phosphorylation in mitochondria by Low *et al.* (*7*). They used various dyes and respiratory inhibitors. It is hard to draw conclusions from these results owing to differences the reaction systems involved. The rate of the ATP-*i*P exchange reaction during oxidative phosphorylation was therefore measured by subtracting the decrease in *i*P from iP^{32} incorporated into organic compounds (*1*). This value is always larger in a reaction mixture where there is no electron transport than in one where oxidative phosphorylation occurs in the presence of succinate as oxidizable substrate. Bronk and Kielley (*8*) reported that the exchange reaction in preparations of mitochondria prepared by supersonic disintegration is stimulated by reduction of the electron carriers. However Wadkins and Lehninger (*6*) found that the exchange was inhibited if the carriers were almost completely reduced and suggested that some oxidized carrier, or carriers which were in the process of being oxidized, may be necessary for the reaction.

Addition of various concentration of succinate to a reaction medium containing antimycin A inhibited the exchange (Table II). In support of Lehninger's view (*9*), this results probably shows that reduction of some carrier at the cytochrome b level decreases the exchange activity.

The exchange, as calculated above, which is accompanied with oxidative phosphorylation in the presence of succinate, is increased slightly by addition of cyanide. At the same time there is inhibition of oxidative phosphorylation (Table I). Similarly, the exchange was greater when the reaction took place under nitrogen than under air (Table III).

From these results, it seems that slight reduction of the carriers stimulates rather than inhibits the exchange. When there is slow oxidation-reduction turnover of carriers more "high energy compounds" related to oxidative phosphorylation (ATP-*i*P exchange) are formed than when the carriers are almost completely oxidized.

Furthermore, since a nitrogen atmosphere was more beneficial than air primary high energy bonds are certainly formed with the carriers (*9*) during their conversion from the reduced to the oxidized form.

There was a difference in the pH optima with oxidized and reduced carriers at pH 6.3, 7.2~7.3 and 7.8~8.5 (Fig. 1 3). The three peaks probably correspond to Slater's three pH optima found in the reaction of DNP-activated ATP-ase and in oxidative phosphorylation. Moreover, the peaks in the ATP-*i*P exchange reaction are increased when the carriers are oxidized. These results are in agreement with the observations of Wadkins and Lehninger (*6*).

SUMMARY

1. Using rat liver mitochondria the relation of the ATP-*i*P exchange reaction to oxidative phosphorylation was studied.

2. Extreme reduction of the carriers inhibited the exchange more than oxidation of the carriers.

3. When the carriers were slightly reduced by a low concentration of cyanide or a nitrogen gas phase, the exchange reaction increased probably owing to formation of phosphorylated intermediates.

4. Alternation of the gas phase from nitrogen (preincubation) to air (incubation) enhanced the exchange reaction.

5. As with the DNP-activated ATP-ase and oxidative phosphorylation reactions reported by Slater *et al.* (*2*, *3*, *4*), there were three pH optima on the exchange reaction.

6. The height of the optima peaks varied with the oxidation-reduction state of the carriers at each oxidative phosphorylation step.

The author wishes to express his gratitude to Prof. K. Okunuki for his guidance. He would like to thank his colleague Mr. Higashiyama of the same laboratory for helpful discussion during this work.

REFERENCES

(*1*) Iwasa, K., *J. Biochem.*, **47**, 467 (1960)

(*2*) Myers, D. K., and Slater, E. C., *Biochem. J.*, **67**, 558 (1957)

(*3*) Slater, E. C., *Proc. Int. Sym. Enz. Chem.* (*Tokyo-Kyoto*), 288 (1957)

(*4*) Hulsmann, W. C., and Slater, E. C., *Nature*, **180**, 372 (1957)

(*5*) Hagihara, B., Horio, T., Yamashita, J., Nozaki, M., and Okunuki, K., *Nature*, **178**, 629 (1956)

(*6*) Wadkins, C. L., and Lehninger, A. L., *J. Am. Chem. Soc.*, **79**, 1010 (1957): *J. Biol. Chem.*, **234**, 681 (1959)

(*7*) Low, H., Siekevitz, P., Ernster, L., and Lindberg, O., *Biochim. et Biophys. Acta*, **29**, 392 (1958)

(*8*) Bronk, J. R., and Kielley, W. W., *Federation Proc.*, **16**, 158 (1958)

(*9*) Lehninger, A. L., Wadkins, C. L., Cooper, C., Davlin, T. M., Gamble Jr., J. L., *Science*, **128**, 450 (1958)

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CHEMICAL DRAG COEFFICIENT OF A STATIONARY ENZYME REACTION IN NEARLY EQUILIBRIUM STATE

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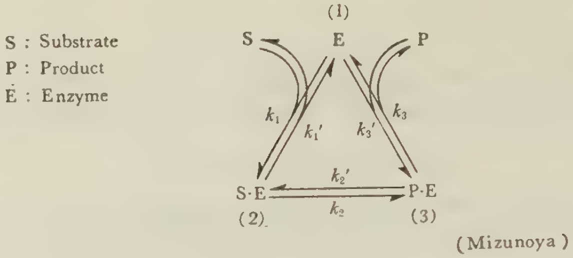
Previously (*1*, *2*), an assumption has been used by the author that each reaction in the metabolizing system proceeds in a nearly equilibrium state and the (enzyme) reaction rate is linearly related to the chemical affinity. This phenomenological (proportional) coefficient is called the chemical drag coefficient or "neck factor" (by M. Sugita) which corresponds to the conductance, *i.e.*, the inverse of the resistance of the reaction. Prigogine (*3*) and de Groot (*4*) *etc.* have considered temporarily that the coefficients in general are constant. In processes *in vivo*, however, the coefficient of enzyme reaction cannot be constant (*5*). It may be a function of the activity of enzyme, which depends on the concentration of hydrogen or metal ion, the amount of activators or inhibitors, or the amount of hormones, *etc.* If a reaction changes the activity of other enzyme, the velocity of this enzyme reaction is influenced by the former reaction. Such a mode of influence through the mediation of the neck factor has been called the "informational correlation" in chemical systems by Sugita (*5*). It is desirable from this viewpoint to express the neck factor as a function of the amount of enzyme. It is the purpose of this paper to clarify the dependence of the neck factor on the amount of enzyme by giving a detailed expression to the chemical drag coefficient of a stationary enzyme reaction in nearly equilibrium state.

THEORETICAL

Denoting by *1* the state of free active site of the enzyme molecule, and by *2* and *3* the states of active site combined with the substrate and product, respectively, the run of enzyme reaction can be expressed as the cyclic transition among the states of the active site; $1 \rightarrow 2 \rightarrow 3 \rightarrow 1$. This transition scheme can also be regarded as the same to the Haldane's "completer scheme of reversible enzyme reaction" (Fig. 1) (*6*).

Let v_1 be the rate of adsorption of the substrate molecule to the active site of enzyme, v_2 be the rate of conversion from the substrate to the product on the surface of the enzyme molecule, and v_3 be the rate of desorption of the product molecule from the active site of enzyme. These rates are defined as the net number of molecules adsorbed, converted and desorbed, respectively, within a unit time interval per unit volume of the reaction



$$S + E \underset{k_1'}{\overset{k_1}{\rightleftharpoons}} S\cdot E \underset{k_2'}{\overset{k_2}{\rightleftharpoons}} P\cdot E \underset{k_3'}{\overset{k_3}{\rightleftharpoons}} P + E$$

(Haldane)

FIG. 1. The scheme of enzyme reaction.

system. Then, denoting by n_i the number of active sites being in the state i ($i=1, 2, 3$), the change of n_i within a unit time interval is written as

$$\begin{aligned}
&dn_1/dt = V(v_3 - v_1),\\
&dn_2/dt = V(v_1 - v_2),\\
&dn_3/dt = V(v_2 - v_3),
\end{aligned}\qquad (1)$$

where V is the total volume of the reaction system. If we denote by n_s and n_p the numbers of the free substrate and product being not adsorbed to the active site of enzyme, respectively, we have

$$\begin{aligned}
&dn_s/dt = -V\cdot v_1,\\
&dn_p/dt = V\cdot v_3.
\end{aligned}\qquad (2)$$

If the mass action law is applied to all the three processes, *i.e.*, the adsorption of substrate to the active site, the conversion from the substrate to the product on the enzyme molecule, and the desorption of product from the active site, the rate of each process is given as

$$\begin{aligned}
&V\cdot v_1 = k_1 n_1 n_s - k_1' n_2,\\
&V\cdot v_2 = k_2 n_2 - k_2' n_3,\\
&V\cdot v_3 = k_3 n_3 - k_3' n_1 n_p,
\end{aligned}\qquad (3)$$

where k_i and k_i' are the forward and backward velocity constants of the rate process of the step i ($i=1, 2, 3$). k_1 and k_3' are different from the others in the point that the dimension is V^{-1}.

If we denote by μ_s and μ_p the chemical potentials of the substrate and the product, respectively, and by μ_1, μ_2 and μ_3 the chemical potentials of the free active site, the substrate- and the product-active site complexes, respectively, the chemical affinity of each process is expressed as

$$\begin{aligned}
&A_1 = \mu_s + \mu_1 - \mu_2,\\
&A_2 = \mu_2 - \mu_3,\\
&A_3 = \mu_3 - \mu_1 - \mu_p.
\end{aligned}\qquad (4)$$

Using the chemical affinity A_i, Eqn. (3) is rewritten as

$$V \cdot v_1 = k_1 n_1 n_s \{1 - \exp(-A_1/RT)\},$$
$$V \cdot v_2 = k_2 n_2 \{1 - \exp(-A_2/RT)\},$$
$$V \cdot v_3 = k_3 n_3 \{1 - \exp(-A_3/RT)\}. \tag{5}$$

If these processes run under the condition of nearly equilibrium state, $|A_i/RT| \ll 1$, Eqn. (5) can be rewritten as

$$v_1 = k_1 n_1 n_s A_1/RTV = L_1 A_1,$$
$$v_2 = k_2 n_2 A_2/RTV = L_2 A_2,$$
$$v_3 = k_3 n_3 A_3/RTV = L_3 A_3, \tag{6}$$

where

$$L_1 \equiv k_1 n_1 n_s/RTV,$$
$$L_2 \equiv k_2 n_2/RTV,$$
$$L_3 \equiv k_3 n_3/RTV. \tag{7}$$

Under the condition of $A_i/RT \ll 1$, the velocity is linearly related to the chemical affinity. The coefficient L_i, though, is not constant but depends on the number of active sites of the enzyme molecule. After Prigogine, n_i and n_s in Eqn. (7) are approximately equal to the corresponding values in the equilibrium state, because the reaction proceeds in nearly equilibrium state (*3*). Exactly, however, the n_i and n_s should not take the equilibrium values even in nearly equilibrium state.

Now, we have $dn_i/dt = 0$ in the stationary state. Applying this relation to Eqn. (1), we obtain

$$v_1 = v_2 = v_3 \equiv v. \tag{8}$$

If we denote by A the overall chemical affinity of this enzyme reaction, we can write as

$$A \equiv \mu_s - \mu_p = \sum_{i=1}^{3} A_i. \tag{9}$$

Rewritting Eqn. (6) and using the relations (8) and (9), we have

$$A = v \sum_{i=1}^{3} (1/L_i), \tag{10}$$

or we obtain a linear relation between the chemical affinity and the velocity of the stationary enzyme reaction as follows

$$v = L \cdot A \tag{11}$$

where

$$\frac{1}{L} \equiv \sum_{i=1}^{3} \left(\frac{1}{L_i} \right). \tag{12}$$

Using the relation (7), Eqn. (12) is rewritten as

$$\frac{1}{L} = RTV \left(\frac{1}{k_1 n_1 n_s} + \frac{1}{k_2 n_2} + \frac{1}{k_3 n_3} \right). \tag{13}$$

In the stationary state, the number of the active sites being in the state i ($i = 1, 2, 3$) is derived from Eqns. (3) and (8) as follows

$$n_1 = (k_2 k_3 + k_3 k_1' + k_1' k_2') \, n/k^2,$$
$$n_2 = \{(k_3 + k_2') \, k_1 n_s + k_2' k_3' n_p\} \, n/k^2,$$
$$n_3 = \{k_1 k_2 n_s + (k_2 + k_1') \, k_3' n_p\} \, n/k^2, \tag{14}$$

where n is the total number of the active sites, that is

$$n = \sum_{i=1}^{3} n_i, \tag{15}$$

and

$$k^2 = k_2k_3 + k_3k_1' + k_1'k_2' + (k_2 + k_3 + k_2')\, k_1n_s + (k_2 + k_1' + k_2')\, k_3'n_p. \tag{16}$$

These expressions were early given by Haldane (*6*).

Inserting the stationary values of the number of active sites expressed as Eqn. (14) into Eqn. (13), we obtain

$$L = \frac{k_1\,k_2\,k_3\,n_s\,n}{\lambda\,k^2\,RTV} \tag{17}$$

where

$$\lambda \equiv \frac{k_2k_3}{k_2k_3 + k_3k_1' + k_1'k_2'} + \frac{k_1k_3\,n_s}{(k_3 + k_2')\,k_1n_s + k_2'k_3'n_p} + \frac{k_1k_2\,n_s}{k_1k_2n_s + (k_2 + k_1')\,k_3'n_p}. \tag{18}$$

On the other hand, the stationary velocity of the enzyme reaction is derived from Eqns. (3), (8) and (14) as follows

$$v = \left(\frac{k_1k_2k_3\,n_s - k_1'k_2'k_3'\,n_p}{k^2}\right)\frac{n}{V}. \tag{19*}$$

Thus the condition of chemical equilibrium of the enzyme reaction is given by

$$k_1k_2k_3\,n_s = k_1'k_2'k_3'\,n_p. \tag{20}$$

Under this condition, we obtain $\lambda = 1$ from the relation (18). Since the velocity constants k_i and k_i' and the concentrations of the substrate and product are all positive definite, λ is not greater than three. If all the velocity constants of the backward reactions are zero, λ takes the maximum value three. This corresponds to the case of infinite equilibrium constant. In other words, the enzyme reaction is completely irreversible and uni-directional catalysis. Thus, instead of the equilibrium constant, λ may be regarded as a measure of the irreversibility of the enzyme reaction.

CONCLUSION

The velocity of a stationary enzyme reaction in nearly equilibrium state is given as follows

$$v = \frac{\prod_i k_i\,n_s\,n}{\lambda\,k^2\,RTV}\cdot(\mu_s - \mu_p)$$

with the notations;

μ_s: the chemical potential of the substrate, μ_p: the chemical potential of the product, n: the total number of the active sites of enzyme, n_s: the number of the free substrate molecule, n_p: the number of the free product molecule, V: the total volume of the reaction system, R: the gas contsant, T: the absolute temperature, k_i: the velocity constant of the forward process of the step i $(i = 1, 2, 3)$, k_i': the velocity constant of the backward process of the step i $(i = 1, 2, 3)$. Both k and λ are the functions of k_i, k_i', n_s and n_p defined as Eqns. (*16*) and (18), respectively.

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* This equation is also obtained by rewritting the Haldane's equation.

Above equation shows that the neck factor of an enzyme reaction depends on the amount of enzyme. This may throw the light on the phenomenological analysis of the metabolism by means of the idea of "informational correlation".

SUMMARY

A detailed expression is given to the chemical drag coefficient of the stationary enzyme reaction in nearly equilibrium state. It is clarified in the expression that the coefficient, *i.e.*, the neck factor depends on the amount of enzyme.

The author wishes to express his hearty thanks to Prof. M. Sugita of Hitotsubashi University for his valuable advices in preparation of the manuscript. The author's thanks are also due to Prof. K. Oomori of this laboratory for his encouragement and interest in this work.

REFEREENCES

(*1*) Mizunoya, T., *J. Biochem.*, **46**, 535 (1959)
(2) Mizunoya, T., *J. Biochem.*, **46**, 669 (1959)
(3) Prigogine, I., "Etude thermodynamique des phénomènes irréversibles," Dunod, Paris (1947)
(4) de Groot, S. R., "Thermodynamics of Irreversible Processes," North-Holland Publ. Co., Amsterdam (1951)
(*5*) Sugita, M., *Symposium on Biophysics and Biophysical Chemistry* (in Japanese), **4**, 113 (1959)
(*6*) Haldane, J. B. S., "Enzymes", Longmans, Green and Co., London, p. 81, (1930)

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EFFECTS OF FLAVINS ON THE RESPIRATION OF RAT LIVER MITOCHONDRIA

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(Received for publication, October 19, 1959)

FMS*, which had been synthesized in our laboratory, was found to be a specific competitor with FAD in D-amino acid oxidase system. Consequently, this inhibitor was reasonably expected to be a specific inhibitor for dissociable flavin enzymes.

Using this inhibitor, the authors intended to examine the inhibition of flavin enzyme system existed in the mitochondria, in which the respiratory enzyme system including flavin enzymes is organized. By this examination, the state of the dissociation of the flavin from its apo-enzyme in the mitochondria was expected to be demonstrated. For the same purpose, other flavin derivatives were also utilized.

MATERIALS AND METHODS

FMS was synthesized by sulphation of riboflavin dissolved in pyridine using chlorosulphonic acid at 0° and then at 40°, in dry conditions. The FMS was purified by powdered cellulose chromatography, and finally the calcium salt of FMS was obtained as yellow crystals (*1*). FAD was prepared by the method of Yagi *et al.* (*2*). Its purity was more than 92 per cent, and other flavins, nucleic acids or metals were not present. The sodium salt of FMN and riboflavin were chromatographically purified from commercial samples. ATP was furnished from Schwarz Laboratories Inc., New York, as its sodium salt.

Albino rats weighing about 100 g. were used as experimental animals. Isolation of rat liver mitochondria was carried out according to the method of Schneider and Hogeboom (*3*).

Flavin enzymes contained in α-ketoglutarate, succinate and malate oxidation systems and D-amino acid oxidase were examined by using a conventional Warburg manometer. For the study of α-ketoglutarate, succinate and malate oxidation, the following reaction mixture was used: 2.5×10^{-2} M substrate, 2.5×10^{-3} M $MgCl_2$, 2.5×10^{-2} M ATP, 2.5×10^{-2} M KCl, 1×10^{-4} M flavin derivative (in their final concentrations) and the mitochondria suspension (prepared from 0.1 g. of fresh liver) in 1.7 ml. of 5×10^{-3} M phosphate buffer (pH 7.4). Center well contained 0.2 ml. of 20 per cent KOH. After the temperature equilibrium had been reached, flavin derivative in the side bulb was mixed with the contents of the main chamber. Then the oxygen uptake was measured every five minutes for 100 minutes.

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* Abbreviations: FMS, riboflavin-5′-monosulfate; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide.

For the measurement of D-amino acid oxidation, $1.5\times10^{-2}\,M$ D-alanine, $1\times10^{-3}\,M$ KCN, $1\times10^{-4}\,M$ flavin derivative (in their final concentrations) and the mitochondria suspension (prepared from 0.1 g. of fresh liver) in $5\times10^{-2}\,M$ pyrophosphate buffer (pH 8.3) were put into the manometer flask. After the temperature equilibrium had been reached, flavin derivative was mixed from the side bulb, and the readings were taken as mentioned above.

RESULTS AND DISCUSSION

α-Ketoglutarate, Succinate and Malate Oxidation—Using the above mentioned reaction mixture, the oxygen uptake in the case of α-ketoglutarate, succinate or malate was measured. Q_{O_2} (*μl.* O_2 uptake/hour/mg. dried substance of the mitochondria) were 26.9, 33.3 and 18.9 for α-ketoglutarate, succinate, and malate, respectively. The addition of FMS from the side bulb did not inhibit the oxygen uptake by the mitochondria with any of these three substrates. FAD, FMN and riboflavin were also found to have no effect on the oxygen uptake of the mitochondria. These results are shown in Fig. 1.

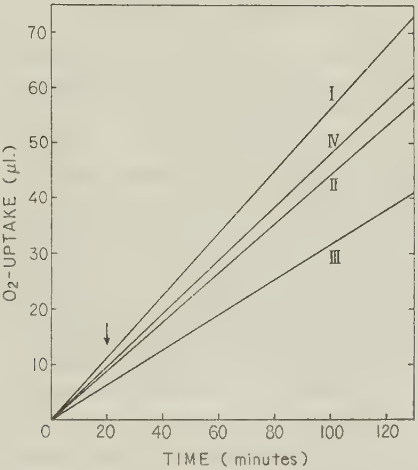

FIG. 1. Effects of flavins on the oxygen uptake of rat liver mitochondria. Reaction mixture I, II or III contains the mitochondria (prepared from 0.1 g. of fresh liver), $2.5\times10^{-3}\,M$ $MgCl_2$, $2.5\times10^{-2}\,M$ ATP, $2.5\times10^{-2}\,M$ KCl, and the substrate mentioned below in 1.7 ml. of $5\times10^{-3}\,M$ phosphate buffer (pH 7.4). I: With succinate ($2.5\times10^{-2}\,M$). II: With α-ketoglutarate ($2.5\times10^{-2}\,M$). III: With malate ($2.5\times10^{-2}\,M$). Reaction mixture IV contains the mitochondria (prepared from 0.1 g. of fresh liver), $1\times10^{-3}\,M$ KCN, $1.5\times10^{-2}\,M$ D-alanine in 1.7 ml. of $5\times10^{-2}\,M$ pyrophosphate buffer (pH 8.3). To these reaction mixtures, FAD, FMN, riboflavin or FMS ($1\times10^{-4}\,M$, in their final concentration) was added from the side bulb of manometer flask, as shown by an arrow. The oxygen uptake per mg. dry weight of the mitochondria was shown. The results with and without the addition of any flavin agreed on the same curve in every experiment.

Concerning FAD, Eggleston and Williamson (*4*) reported that FAD ($10^{-5}-10^{-4}$ *M*) inhibits the oxygen uptake of the liver suspensions with α-ketoglutarate.

Re-examination on the effect of FAD was carried out by using the liver suspensions. It was found that FAD gives no effect on the oxygen uptake of liver suspensions with α-ketoglutarate.

D-Amino Acid Oxidase—By the reaction mixture mentioned above, the activity of D-amino acid oxidase was tested. As shown in Fig. 1, FMS and other flavins gave no effects on the oxygen uptake of liver mitochondria with D-alanine.

Incubation of the Mitochondria With Flavins—By the incubation of the mitochondria with flavin at 4° for three hours, FMS and other flavins had no effects on the oxygen uptake of liver mitochondria not only with α-ketoglutarate but also with D-alanine.

These results might inform as to the actual state of the existence of flavins in the mitochondria. If flavin enzymes in the mitochondria were in such freely dissociable state as purified D-amino acid oxidase, the added FAD would increase the activities of flavin enzymes which have FAD as coenzyme, and the added FMS would inhibit them. Nevertheless, no effects of flavins were found on the oxygen uptake of the mitochondria. So, it might be considered that flavins in the mitochondria would exist in such a state as the added flavins could not replace them.

Similar conclusions had been made by Huennekens and Green (*5*) concerning pyridine enzyme. There is a considerable amount of bound DPN and TPN in both rabbit liver and kidney mitochondria. This bound form is much more stable than added pyridine nucleotide. Potter (*6*), in his studies on rat liver homogenates, also came to a similar conclusion about the excess of diaphorase. Addition of diaphorase to the mitochondria did not increase the rate of reduction of 2,6-dichlorophenolindophenol. Furthermore, diaphorase did not appear to be split to any significant extent by the various procedures which were used to split the pyridinoprotein oxidases of the cyclophorase complex.

However, Huennekens and Green (*7*) further studied on pyridine enzyme in the mitochondria, and found that after the mitochondria has been aged at 0° for some days, various enzymes which previously showed no requirement for pyridine nucleotide become partially or completely dependent for their activity upon the addition of pyridine nucleotide. Evidence was presented that these treatments lead to the splitting out of bound pyridine nucleotide and to the transition from conjugated to dissociated pyridinoprotein enzymes.

So, the correlation between aged mitochondria and flavin derivatives was studied.

Aged Mitochondria—The mitochondria prepared from rat liver were aged at 4° for 5 days. The oxygen uptake of the aged mitochondria with D-alanine and the effects of flavins on it were examined. The results are shown in Fig. 2. As shown in the figure, the oxygen uptake of aged mitochondria markedly decreased comparing with fresh mitochondria. However, differing from fresh mitochondria, the addition of FAD to the reaction mixture increased the oxygen uptake of the mitochondria, while the addition of FMS decreased it. The similar results were obtained for the respiration of aged mitochondria with α-ketoglutarate. So, it might be considered that flavins in the aged mitochondria would exist in such a state as the added flavins could replace them.

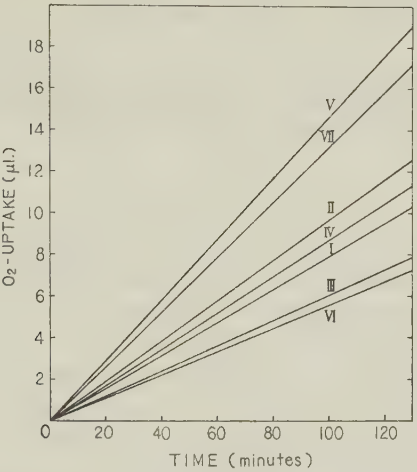

Fig. 2. Effects of flavins on the oxidation of D-alanine by aged mitochondria of rat liver. The reaction mixture contains the same components as those of IV in Fig. 1 except flavin. The conditions of the mitochondria and flavin were as follows: I: The mitochondria aged at 4° for 5 days. II: I+1×10^{-3} *M* FAD (final concentration). III: I+1×10^{-3} *M* FMS (final concentration). IV: The mitochondria aged with FAD (1×10^{-3} *M*) at 4° for 5 days. V: After the aging of IV, 1×10^{-3} *M* FAD (calculated as its final concentration) was further added. VI: The mitochondria aged with FMS (1×10^{-3} *M*) at 4° for 5 days. VII: After the aging of VI, 1×10^{-3} *M* FAD (final concentration) was added. The oxygen uptake per mg. dry weight of the mitochondria was shown.

Aging with FAD—The mitochondria were aged with FAD at 4° for 5 days. Then, FAD was further added to the reaction mixture to saturate FAD for the enzyme protein in the mitochondria. The oxygen uptake was larger than that of the mitochondria aged without FAD and then saturated with FAD as shown in Fig. 2.

Aging with FMS—The mitochondria were aged with FMS at 4° for 5 days. Then, FAD was added to saturate the enzyme protein in the mitochondria, the oxygen uptake was larger than that of the mitochondria aged without flavin and then saturated with FAD as shown in Fig. 2.

According to Burton (*8*), at pH 8.25 and at 37.5°, FAD, substrates and the competitive inhibitors, L-leucine and sodium benzoate, protect D-amino acid oxidase apo-enzyme from thermal inactivation.

So, it might be considered that FAD would protect D-amino acid oxidase apo-enzyme from inactivation during the aging of the mitochondria. The fact that FMS protect the apo-protein of flavin enzyme in the aged mitochondria may be attributed to the complex formation of the inhibitor and the apo-protein.

These results indicate that in the aged mitochondria, differed from fresh mitochondria, the state of flavin enzymes was changed so as to be able to replace the co-enzyme for its analogue.

SUMMARY

1. FMS, the specific inhibitor of flavin enzymes, did not affect the oxygen uptake of freshly prepared mitochondria of rat liver with α-ketoglutarate, succinate, malate, and D-alanine.

No effects of flavins (FAD, FMN, riboflavin and FMS) on the oxygen uptake of the mitochondria were found, even if incubated with these flavins at 4° for three hours.

Against the report of Eggleston and Williamson, FAD did not inhibit the oxygen uptake of liver suspensions.

These results indicate that the flavins in the mitochondria exist in compact bound form.

2. On the other hand, by aging the mitochondria at 4° for 5 days, added FAD increased the oxygen uptake and added FMS decreased it.

These results suggest that flavins in the aged mitochondria would exist in such a state as the added flavins could replace them.

3. By aging the mitochondria, apo-protein of flavin enzyme in the mitochondria is partially inactivated. The coexistence of FAD, even FMS, protects the inactivation of the apo-protein during the aging.

REFERENCES

(*1*) Takahashi, N., Yagi, K., and Egami, F., *J. Chem. Soc. Japan, Pure Chem. Sect.*, **78**, 1287 (1957)
(*2*) Yagi, K., Matsuoka, Y., Kuyama, S., and Tada, M., *J. Biochem.*, **43**, 93 (1956)
(*3*) Schneider, W. C., and Hogeboom, G. H., *J. Biol. Chem.*, **183**, 123 (1950)
(*4*) Eggleston, L. V., and Williamson, D. H., *Biochem. J.*, **56**, 250 (1954)
(*5*) Huennekens, F. M., and Green, D. E., *Arch. Biochem.*, **27**, 418 (1950)
(*6*) Potter, V. R., *J. Biol. Chem.*, **165**, 311 (1946)
(*7*) Huennekens, F. M., and Green, D. E., *Arch. Biochem.*, **27**, 428 (1950)
(*8*) Burton, K., *Biochem. J.*, **48**, 458 (1951)

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PHOSPHORUS METABOLISM IN HUMAN ERYTHROCYTE

II. SEPARATION OF ACID-SOLUBLE PHOSPHORUS COMPOUNDS INCORPORATING P^{32} BY COLUMN CHROMATOGRAPHY WITH ION EXCHANGE RESIN*

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(Received for publication, October 21, 1959)

In the preceding paper (*1*) the authors have described the use of paper chromatographic procedure in order to obtain an information about the compounds participating in phosphorus metabolism in human red cells. In the course of further experiments it became necessary to obtain more accurate analytical values of these compounds. Paper chromatography is, though rapid and simple, not satisfactory for the purpose, but the application of column chromatography on ion exchange resin has been proved helpful in the separation and quantitization of the compounds taking up radioactive isotopes. Bartlett *et al.* (*2*) was the first to separate and identify some glycolytic intermediates in human erythrocyte with the aid of Dowex 1-chloride column, and recently Bartlett (*3*) published an elaborate investigation on this subject.

The present communication deals with the experiments done in the authors' laboratory for some years concering the incorporation of P^{32} into the acid-soluble phosphorus compounds in human erythrocyte by the use of ion exchange resin chromatography.

EXPERIMENTALS

Blood was drawn by venipuncture from young healthy men with added heparin as anticoagulant (1 mg. per 100 ml.). The blood was shaken gently in a water bath at 37°

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Abbreviations: AMP, adenosine 5′-monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; Atetra P, adenosine tetraphosphate; IMP, inosine 5′-monophosphate, DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; PGA, phosphoglyceric acid; DPGA, diphosphoglyceric acid; G-6-P, glucose 6-phosphate; F-6-P, fructose 6-phosphate; G-1, 6-DP, glucose 1, 6-diphosphate; F-1, 6-DP, fructose 1, 6-diphosphate; SMP, sugar monophosphate; HDP, hexose diphosphate; *i*P, inorganic orthophosphate.

for 30 minutes with the gas phase of air containing 5 per cent CO_2 to assure the steady state of cell metabolism the pH, being kept constant between 7.40 and 7.45. At the end of preincubation period was added a small volume of solution of P^{32} in isotonic NaCl containing 0.04 mg. carrier P per ml. as sodium phosphate bufferred at pH 7.4. The incubation was continued further for a given period of time, followed by washing of the erythrocytes and removal of 'buffy coat' in the same way as described previously (*1*). In some experiments, removal of leucocytes and platelets was carried out as follows prior to incubation. The blood was centrifuged at $1300 \times g$ for 20 minutes, the plasma pipetted out and set aside, the top layer of the cells aspirated off, and the packed cells were suspended in the plasma. The cells were packed again by centrifugation, the top layer of the cells was drawn off and discarded, and the red cells were again suspended in the plasma.

Much care was taken with reference to purity of radioactive orthophosphate to be added to the red cells. The original solution of P^{32} specified as H_3PO_4 in HCl supplied by the Radiochemical Centre, Amersham, England occasionally contained a slight but not negligible amount of P^{32} other than orthophosphoric acid (*4*). The interference by this contaminant could be minimized by heating the original solution which contained a sufficient amount of HCl in a boiling water bath for one hour or two with added carrier orthophosphoric acid immediately before use.

The red cells incubated as described above were deproteinized with either trichloroacetic acid or perchloric acid according to the type of subsequent chromatography.

Two systems of column chromatography were employed in the present study; chromatography with Dowex 1-chloride developed by Cohn and Carter (*5*) and with Dowex 1-formate devised by Hurlbert *et al.* (*6*).

Dowex 1-Chloride Chromatography—Thirty milliliters of blood was pipetted into an ice-cold 20 per cent trichloroacetic acid (TCA) of the equal volume. The subsequent operations were carried out in a cold room kept at near 2°. The alcohol-insoluble barium salt was precipitated from the TCA extract as described previously (*1*) and decationized by treating with ion exchange resin as follows. The barium salt obtained from 30 ml. of blood was suspended in a small amount of water, treated with 3 ml. of Amberlite 120 (H^+ form) for more than 10 minutes, and 15 ml. of water was added. The supernatant fluid after centrifugation was transferred into a vessel, the resin was washed twice with water, and the washings were combined with the supernatant. The fluid was diluted to 150 ml. with water, passed through an Amberlite 120 (H^+) column of $0.8\ cm.^2 \times 5\ cm.$ at a flow rate of about 1.5 ml. per minute and was followed by water to wash through the column. To the effluent were added 5 ml. of KH_2PO_4 solution (1 mg./ml.) and water to make up to 800 ml., and neutralized to pH 8 with ammonia. No attempt was made to analyze the alcohol-soluble fraction in this case, since very little incorporation of P^{32} had been observed in this fraction in the preliminary experiments.

The decationized and neutralized solution was run through Dowex 1-chloride (X-2, 200-400 mesh) at a flow rate of about 1.5 ml. per minute, a resin bed of $0.5\ cm^2 \times 7.0\ cm.$ being used and followed by 200 ml. water. It is advisable to wash the resin previously with several volumes of acetone to remove ultraviolet-absorbing contaminants. The column was then treated with 1000 ml. of a solution of $M/40$ ammonium chloride and $M/100$ potassium tetraborate to remove inorganic phosphate (*7*), followed by washing with water. If complete removal of *i*P was needed, 4 mg. of carrier P as potassium phosphate solution of pH 8 was put again into the column and the same eluting solution was passed through. Glucose 1-phosphate may be lost by this procedure but the presence of this ester in human erythrocyte is not demonstrated (*1*, *3*). The subsequent elution was carried out according to the method of Cohn and Carter (*5*) with minor modifications. The compounds absorbed on the resin were eluted at a flow rate of 1.5 ml. per minute, collecting 10 ml. fractions in the following sequence: 5/1000 N HCl, N/100 HCl, N/100 HCl $+M$/50 NaCl, N/100 HCl$+M$/25 NaCl, N/100 HCl$+M$/15 NaCl, N/100 HCl$+M$/4 NaCl. If the eluates were to be kept for further examinations they were neutralized with ammonia.

Dowex 1-*Formate Chromatography*—To 1 volume of erythrocyte suspension or blood were added 2 volumes of 0.8 N perchloric acid (PCA) and the mixture was centrifuged at $1300\times g$ for 20 minutes. In most cases, washing of the protein residue was omitted to avoid increasing the volume of the extract. The efficiency of the single extraction by this procedure was about 80 per cent as determined by the recovery of P^{32} added to the cold cell suspension immediately before deproteinization. Correction of the analytical values was conducted on the basis of this recovery test. The extract obtained from 30ml. blood or an equivalent amount of blood cells was neutralized with KOH to pH 6 to 7, filtered or centrifuged to remove $KClO_4$, and condensed to a small volume less than 20ml. by lyophilization. Precipitated $KClO_4$ was again centrifuged off in the cold. The supernatant solution was diluted with 300 to 500 ml. of water and run through a Dowex 1, X-10, formate column of $0.8\,cm^2\times 15\,cm$. Elution was performed by a slight modification of the method described by Hurlbert *et al.* (*6*). Usually, the fractions of 10 ml. volume were collected.

The optical density of the fractions of the column chromatograms was read at 260 mμ. and, if necessary, at other wavelengths with a Hitachi Model EPU spectrophotometer with water as the blank. For assay of the radioactivity, 0.5 ml. of each fraction was transferred to a shallow dish, dried under a heater lamp, and counted with a conventional end-window Geiger-Müller counter.

Rechromatography—Paper chromatography was employed for further resolution and identification of the individual compounds. The fractions covering a peak from the chloride resin chromatogram were pooled, neutralized with ammonia, diluted, passed through a Dowex 1-chloride column of a smaller size and the absorbed anions were eluted with a small volume of 0.2 N HCl. The fractions from the formate column were evaporated *in vacuo* over KOH. In either case, the concentrate obtained was neutralized, phosphates were precipitated by addition of 20 per cent barium acetate and four volumes of ethanol, decationized with Amberlite IR 120 (H^+) and submitted to paper chromatography (*1*). The following developing solvent mixtures were used: A, isobutyric acid-0.5 N ammonia (20 : 12) (*8*); B, isopropanol-isoamyl alcohol-5 per cent TCA-72 per cent lactic acid (15 : 5 : 10 : 0.5) (*9*) and C, *tert*-butanol-picric acid-water (80 : 4 : 20) (*10*). The paper chromatograms were radioautographed on Fuji No-Screen X-Ray Film, No. 200. The identification of the individual radioactive spots and ultraviolet absorbing spots were made as described in the previous works from the authors' laboratory (*1*, *11*).

The criteria for the identification of the compounds were based on the ultraviolet absorption spectra at different pH, the position of appearance on ion exchange resin chromatograms, behavior in paper chromatography (*1*), the rate of acid hydrolysis, the content of phosphorus and ribose, and the specific color reactions for sugars.

Liberation of radioactive *i*P from the substances by acid hydrolysis was followed by the isobutanol extraction method (*12*). The following color reactions were employed for the quantitative or semi-quantitative analyses: orcinol reaction for pentose by the method of Mejbaum (*13*) in which the heating was prolonged to 40 minutes, resorcinol reaction (*14*) and cysteine carbazol reaction (*15*) for ketohexose, cysteine sulfuric acid reaction for hexose (*16*) and naphthoresorcinol reaction for glyceric acid (*17*).

Ultraviolet absorbing compounds were determined spectrophotometrically, adopting the following molecular extinctions at 260 mμ. at pH 2.0 to 4.0: AMP, 14,600; ADP, 14,500; ATP, 14,300; IMP, 7,700; xanthine, 10,200 (at 267 mμ.), ; uric acid, 11,500 (at 283 mμ.); DPN and TPN, 18,500.

For separation of nucleotide from non-nucleotide phosphorus compounds, the compound was trapped on a column of Norti A (SX-30) activated and treated with octanol (*18*) followed by elution with 0.2 N NH_4OH in 30 per cent ethanol. Labile phosphate groups of ATP and ADP were determined after liberation of P by adenosine triphosphatase and myokinase (*19*).

Phosphorus was determined by a slight modification of Horecker's method (*20*). Chemical determination of uric acid was performed by the method of Newton (*21*).

RESULTS

Dowex 1-Chloride Chromatography—Fig. 1 represents the results obtained by chromatography of acid-soluble shosphorus compounds of human erythrocytes which were incubated for 30 minutes with P^{32} on a column of Dowex 1-chloride.

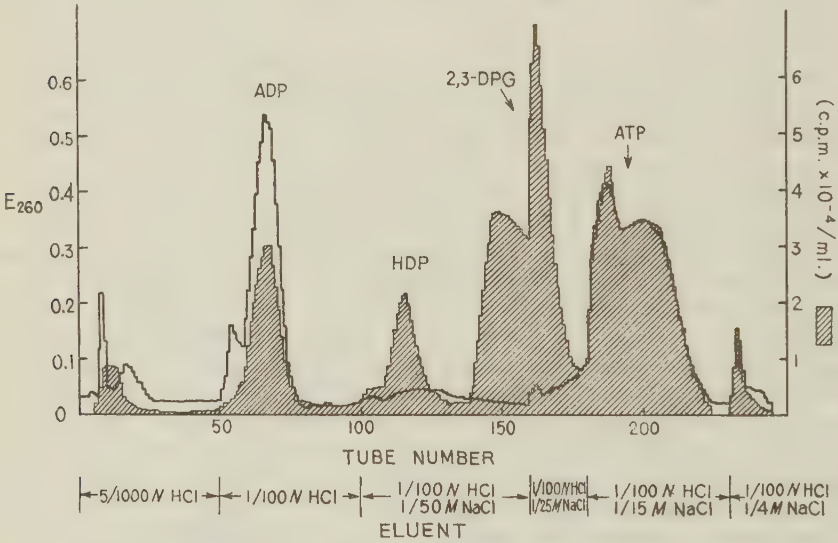

FIG. 1. Chromatography of the acid-soluble phosphorus compounds of human blood by the chloride system. Thirty ml. of blood was incubated with P^{32} at 37° for 30 minutes. Leucocytes and platelets were removed prior to incubation in this experiment. After incubation the blood was extracted wirh TCA. Alcohol-insoluble barium salt of phosphates was precipitated from the extract, decationized with Amberlite 120 (H^+), and submitted to chromatography on a 0.5 cm.^{2} × 7 cm. column of Dowex 1-chloride, X-2 (200 to 400 mesh). Inorganic phosphate was eluted first by passing through M/40 NH_4Cl+M/100 $K_2B_4O_7$. The subsequent eluting solutions are given under the abscissa. The optical density is represented by empty, and radioactivity by cross-hatched areas.

The optical density at 260 mμ. and the radioactivity of P^{32} are shown for the successive fractions. The section taken are described below in the order of their elution.

5/1000 *N* HCl section. One of the two ultraviolet absorbing peaks was AMP which gave the same paper chromatograms run in different solvent mixtures as authentic AMP. The radioactive compounds in this section contained G-6-P and F-6-P which were identified by their characteristic color reactions and paper chromatograms.

TABLE I

Chemical Assays on Adenine Nucleotides from Erythrocytes

Assays were conducted on peak tubes from the chromatogram of chloride system. AtetraP obtained from the *N*/100 HCl+*M*/4 NaCl section was separated from ATP by repeating the same chromatograph on a Dowex 1-chloride column and treated with a Nort A column to remove the non-nucleotide contaminants.

Compound	Adenine (260 mμ)	Ribose	Total P	Labile P
	μmoles	μmoles	μmoles	μmoles
ADP	0.060	0.065	0.132	0.068
ATP	0.055	0.058	0.154	0.103
Atetra P-like nucleotide	0.072	0.084	0.286	0.162

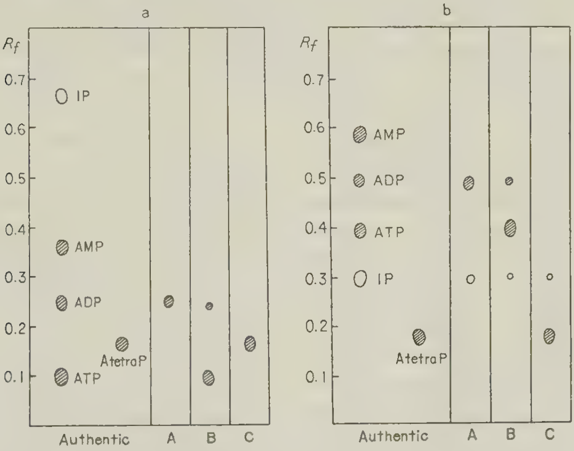

FIG. 2. Paper chromatograms of ADP, ATP and AtetraP.

A hundred ml. of human blood was incubated with 1 mc. of P^{32} at 37° for 30 minutes and acid-soluble phosphates in the TCA extract of the blood were chromatographed on a Dowex 1-chloride column. Leucocytes and platelets were removed after the incubation. A, B and C are the paper chromatograms of the adenine nucleotide peak fractions obtained from the column by elution with *N*/100 HCl, *N*/100 HCl+*M*/25 NaCl and *N*/100 HCl+*M*/5 NaCl, respectively.

Radioactivity, empty areas; ultraviolet absorption, cross-hatched areas. Developing solution: a, isopropanol-isoamyl alcohol-TCA-lactic acid; b, isobutyric acid-ammonia.

N/100 HCl section. The figure shows two ultraviolet absorbing peaks, one small and the other large. The latter was proved to be ADP from the analytical data on the peak tube in which the ratio, adenine (optical density at 260 mμ.) to ribose (orcinol) to total phosphorus to labile phosphorus (hydrolysis in 1 N H_2SO_4 at 100° for 10 minutes) was approximately 1 : 1 : 2 : 1 (Table I), and the paper chromatograms with the same R_f values as those of authentic ADP. The smaller peak which appeared ahead of ADP was suggested to be IMP by rechromatography on paper.

The radioactive peak was in agreement with the ADP peak in its position on column chromatograms. The paper chromatograms of the eluate of this section showed distinct radioactive spots corresponding to 3-PGA and other unknown compounds in addition to the strongly radioactive spot of ADP.

N/100 HCl+M/50 NaCl section. The radioactive peaks contained F-1, 6-DP which developed a red color by heating with resorcinol and HCl. The paper chromatograph of this fraction gave a spot with R_f value of F-1, 6-DP but contained another phosphorus compound as indicated by the ratio, phosphate to fructose, which was much greater than 2 : 1. The major constituent of this fraction was identified as glucose 1,6-diphosphate as described below.

N/100 HCl+M/25 NaCl section. 2,3-DPGA was eluted in this section, developing a characteristic color with naphthoresorcinol. The diphosphate was eluted partly in the preceding section as illustrated in Fig. 1 when the elution with N/100 HCl+M/50 NaCl was continued beyond completion of the elution of hexose monophosphate.

N/100 HCl+M/15 NaCl section. The peak fractions which were collected in this section of the eluate gave approximately a 1 : 1 : 3 : 2 ratio of adenine to ribose to total phosphorus to labile phosphorus (Table I) and the same ultraviolet spectrum as authentic ATP. Furthermore, the paper chromatograms run in two solvent systems gave main spots corresponding to ATP and accessory spots of ADP and iP which probably originated from ATP by spontaneous decomposition during the manipulation (Fig. 2). The specific activity (radioactivity count/optical density at 260 mμ.) of the successive fractions past the completion of elution of 2,3-DPGA remained constant until the radioactivity leveled off. This fact indicates that ATP is the sole compound existing in these fractions. Occasionally, the peak of ATP appered in two-phasic form as in Fig. 1, the reason being unclarified.

N/100 HCl+M/4 NaCl section. The ultraviolet absorption of this section was attributed to the residue of ATP and another adenine compound which possessed a typical ultraviolet spectrum of adenine nucleotide and gave a radioactive and ultraviolet absorbing spot with R_f value of 0.17 on the paper chromatogram run in the solvent mixture B. For further examination of this new nucleotide, the pooled fractions of this section from 1 liter human blood were rechromatographed on Dowex 1-chloride column, the compound in N/100 HCl+M/5 NaCl section was concentrated with the use of a small Dowex 1-chloride column, eluted with 0.2 N HCl, and treated with Norit A after neutralization to remove non-nucleotide contaminants as described above. Eight micromoles of purified material on the basis of adenine were thus obtained. The absorption maxima determined in 0.1 *N* HCl and in 0.1 *N* KOH were identical with those of other adenine nucleotides. Analytical values are given in Table I for this sample. The ratio, adenine to ribose to total P to labile P, was close to 1 : 1 : 4 : 2. The new nucleotide seems to be closely related to adenosine tetraphosphate described by Marrian (*22*) and Lieberman (*23*). The difference observed so far is in the ratio, total phosphorus to labile phosphorus, which has been estimated for authentic adenosine tetraphosphate* as 4 : 3. R_f values on the paper chromatograms run in the solvent systems A and B were in good agreement between the compound isolated and the authentic sample (Fig. 2). A possibility that the fourth phosphate would be attached to 2 or 3 charbon atom of pentose was ruled out by the evidence that the nucleotide possessed vicinal hydroxyl groups as determined by periodate oxidation according to Dixon and Lipkin (*24*). The identity of ultraviolet spectra of this nucleotide and authentic adenosine tetraposphate makes it appear unlikely that attachment is to the purine ring. The nucleotides isolated from the erythrocytes which had been incubated with P^{32} gave the ratio of the specific activity as expressed in P^{32}/adenine, ADP, 1.0, ATP, 2.0, and the new nucleotide, 1.8.

Dowex 1-Formate Chromatography—In Fig. 3 is illustrated the chromatogram on a colum of Dowex 1-formate of an extract from 30 ml. of human blood incubated with P^{32} for 2 hours. The optical density at 260 mμ. and

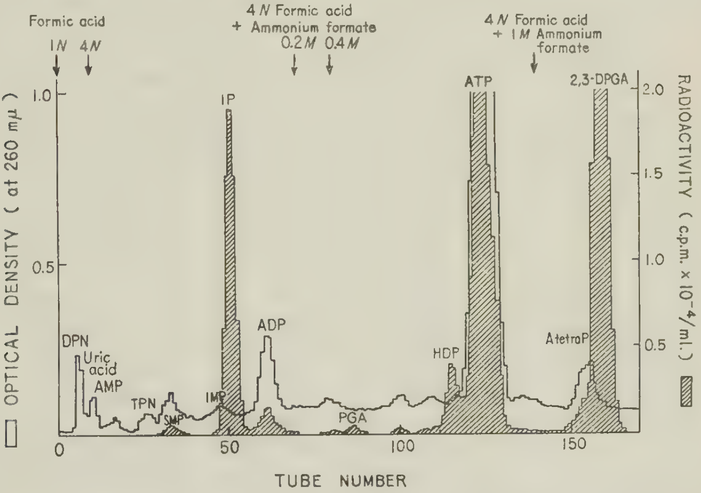

FIG. 3. Chromatography of the acid-soluble phosphorus compounds of human blood by the formate system. Thirty ml. of blood was incubated with P^{32} (120 μc.) at 38° for 2 hours. Erythrocytes were separated from leucocytes and platelets, and extracted with PCA. The compounds were chromatographed on a 0.8 cm.$^2 \times$ 15 cm. column of Dowex 1-formate, X-10, by gradient elution with a mixer of 500 ml. and the solutions mentioned in the figure. Fractions of 10 ml. were collected. Ultraviolet absorption is represented by empty and radioactivity by cross-hatched columns.

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* Obtained from Sigma Chemical Company.

the radioactivity of P^{32} are shown for the successive fractions. The substances found in the fractions are described in the order of their elution.

In the first elution range approaching 1 *N* formic acid, two ultraviolet absorbing peaks were seen. The first compound to appear in the eluate was xanthine, which was identified by its characteristic ultraviolet spectrum with the maximum at 267 mμ. in acid. The fractions with absorption maximum at 260 mμ. came off in succession. The fractions which were pooled and freed from formic acid by evaporation over KOH gave an increase of light absorption at 340 mμ. upon addition to alcohol dehydrogenase system. This suggests that the compound is DPN. The next peak was uric acid which was identified by its ultraviolet spectrum with the maximum at 283 mμ. in acid and at 292 mμ. in alkali.

When the content of the reservoir was changed to 4 *N* formic acid, AMP was eluted at first. The content of AMP in fresh red cells was so low that the exact value could not be obtained. The next peak was suggested to be TPN in its position (*6*) and ultraviolet spectrum.

A small radioactive peak in this region was overlapping sugar monophosphates. When rechromatographed on paper, at least five radioactive spots were observed. Two of them were identified as glucose 6-phosphate and fructose 6-phosphate by mixed chromatography with authentic samples whereas the other three remained unidentified (Fig. 4). Ribose 5-phosphate was very little in amount, if present at all. Acid hydrolysis in 1 *N* H_2SO_4 for 10 minutes liberated only a small portion of the radioactivity of the

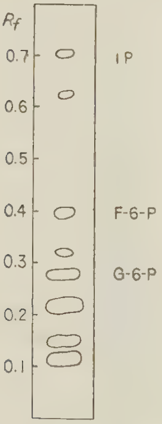

FIG. 4. Rechromatography of sugar monophosphate fractions. Fractions No. 32 to 36 from the sample of Fig. 3 were pooled and evaporated *in vacuo* over KOH. The residue was submitted to paper chromatography run in *tert*-butanol-picric acid-water.

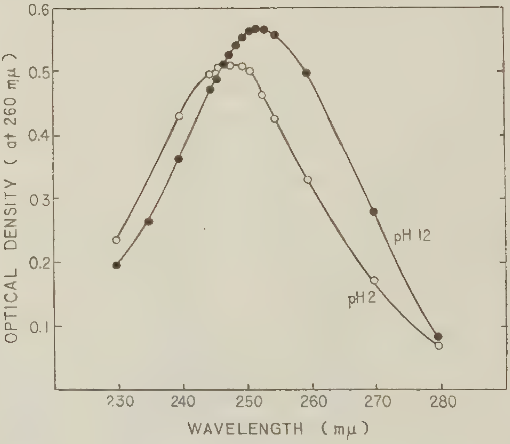

FIG. 5. Ultraviolet absorption spectra of red cell IMP.

The IMP peak from Dowex 1-formate separation (Fig. 3) was used for spectral assay after removal of formic acid.

fractions as *i*P. This suggests that sugar esters with a phosphate group at the position 1 is almost absent as indicated previously (*1*).

An overlapping mixture of *i*P and a nucleotide came off the column next. The fractions were absorbed on Dowex 1-chloride, X-2, column after removal of formic acid, *i*P was washed out by passing through $M/40$ NH_4OH $+M/100$ $K_2B_4O_7$ and the nucleotide was eluted with $N/100$ HCl. The ultraviolet spectra of this substance in acid and alkali showed typical curves of IMP (Fig. 5). The assay values of phosphorus and ribose in reference to the absorbancy at absorption maximum were in good agreement with the theoretical values. The spot obtained on the paper chromatogram run in the solvent system A coincided with that of the authentic sample.

ADP followed *i*P and IMP. Paper chromatography of the peak fractions gave only one spot which corresponded to ADP.

A few small peaks with ultraviolet absorption and with radioactivity appeared between ADP and hexose diphosphate peaks. The compounds with ultraviolet absorption were not identified. As to the radioactive substances, two spots were detected, one having an R_f value a little less than that of *i*P and the other having a larger R_f value, on the paper chromatograms of the fraction, No. 87, of the sample in Fig. 1 run in the solvent C. The former compound was identified as PGA in the same way as described in the previous paper (*1*). The latter seems to be phosphoenolpyruvic acid, since there is the only glycolytic intermediate which possesses an R_f value greater than that of *i*P when run in this solvent.

The P^{32} peak preceding ATP was at the elution position corresponding to hexose diphosphate according to Bartlett *et al.* (*2*), and a more effective separation of this fraction from ATP was achieved by the chromatography on Dowex 1-chloride as described above. P^{32} liberation curve of the compound by acid hydrolysis indicated that two or more phosphate bonds of different nature were contained in the fractions, one of which was labile while the other was relatively stable. The sample was mixed with authentic F-1,6-DP and chromatographed on paper using the *tert*-butanol-picric acid solvent C. Radioautography of the paper chromatogram revealed that a greater portion of the radioactivity was derived from esters different from F-1,6-DP, while the residual smaller portion from F-1,6-DP itself. Further examination of the fractions obtained from a blood sample preserved in acid-citrate-dextrose solution suggested that the former compound was most probably G-1,6-DP as will be reported elsewhere. The existence of G-1,6-DP in human erythrocytes was menthioned by Banderheiden *et al.* (*25*) and, more recenly, proved enzymatically by Bartlett (*3*).

Following the peak of hexose diphosphates, a large peak with ultraviolet absorption and radioactivity of ATP was eluted out. The ratio of radioactivity to optical density at 260 mμ. of the peak fractions was nearly constant. However, a very small unidentified ultraviolet absorbing spot, in addition to ATP and its degradation products, ADP and AMP, was obtained at an R_f value of 0.25 on a paper chromatogram run in the solvent system A.

Another ultraviolet absorbing substance was eluted out following ATP.

It was confirmed that the nucleotide corresponded to Atetra P-like nucleotide found in the elution fractions of Dowex 1-chloride chromatograph.

The last peak with high radioactivity and without ultraviolet absorption gave a single spot on each of chromatograms run in different solvent mixtures. This phosphate ester was proved to be 2,3-DPGA in the same way as described above. Beyond this ester, practically no other phosphorus compund was eluted even when 1 *N* HCl was run through the column.

Concentrations of Erythrocyte Phosphorus Compounds and Distribution of P^{32} among Them—The results of analyses of the acid-soluble phosphorus in fresh human erythrocytes are summarized in Table II. P^{32} distribution among these compounds and their specific activities are presented in Table III.

TABLE II

Analysis of Acid-Soluble Phosphorus Compounds and Their Derivatives in Fresh Human Erythrocytes

Concentrations as μmoles per 100 ml. blood. Exp. 1, the same experiment as given in Fig. 3; Exp. 2, fresh acid-citrate-dextrose blood incubated with P^{32} for an hour; Exp. 3, 4, and 5, whole blood; Exp. 1-5, analyzed by column chromatography on Dowex 1-formate; Exp. 6, analyzed by chromatography on Dowex 1-chloride column.

Compound	Experiment					
	1	2	3	4	5	6
Xanthine	0	0.28	—	—	—	—
DPN fraction1)	2.1	1.4	—	—	—	—
Uric acid	2.8	—	—	—	—	—
AMP	0.3	2.3	—	trace	trace	—
TPN fraction1)	0.6	1.2	0.4	0.65	0.55	—
IMP	0.1	1.1	—	—	—	—
SMP	—	9.2	—	—	—	—
*i*P	—	57.4	—	—	—	—
ADP	2.9	8.1	2.2	5.3	3.7	5.9
PGA	—	1.7	—	—	—	—
HDP (as P)	15.3	12.5	—	—	—	—
ATP	41.2	42.8	41.2	47.9	47.1	42.0
AtetraP-like nucleotide	1.9	2.2	2.1	1.5	1.7	—
2,3-DPGA	159	200	—	—	—	140

1) The values were calculated from the optical density at 260 $m\mu$. assuming that the molecular extinction of the accompanying compounds in acid is 18,500.

The results given in Table II confirm the general observation that 2,3-DPGA is the most abundant phosphate ester in human erythrocytes. Assuming that the hematocrit value is 40 per cent, the concentration of 2,3-DPGA in the cells is 7.0 to 10.0 μmoles P/ml. red blood cells or 21 to 32 mg. P/100 ml.

red blood cells. This is near the value of 7.2 to 10.2 μ moles P/ml. red blood cells reported for 2,3-DPGA in human erythrocytes by Bartlett (*3*) or the average of 28.0 mg. P/100 ml. cells by Rapoport and Guest (*8*). The nucleotide found in the highest concentration in the cells is ATP, which represents over 90 per cent of the total nucleotides except in the Experiment No. 2 of the Table. The concentrations of the nucleotides other than adenine nucleotides are very small, if present at all. As expressed in μ moles P/ml. erythrocytes, ATP amounted to 2.7 to 3.2. Again, these values are close to those reported recently by Bartlett (*3*).

TABLE III

Distribution of P^{32} among Acid Soluble Phosphorus Compounds and Their Specific Radioactivities in Human Erythrocytes

Distribution as per cent of P^{32} incorporated into the acid-soluble P of the cells: relative specific acitivity (R. S. A.) as specific acitivity of each compound divided by that of *i*P. The blood cells were incubated for 30 minutes at 38° after the addition of P^{32}, and the PCA extract was submitted to chromatography on Dowex 1-formate column. The uptake of P^{32} by the cells was 20.9 per cent of the isotope added to the blood.

Compound	P^{32} distribution	R. S. A.
ATP	35.4	α 0.04[1)] β 1.25 γ 1.25
2,3-DPGA	29.2	0.22
*i*P	19.0	1.00
ADP	4.5	α 0.04 β 1.18
HDP	3.0	0.73
AtetraP like nucleotide	2.0	
SMP	0.8	0.26

1) The radioactivity of α-phosphorus was obtained as that of difficultly hydrolyzable P by treating with acid in the way previously reported (*26*). The radioactivity of β- and γ-phosphorus was calculated assuming that a very rapid equilibrium is reached among labile phosphate groups of ATP in human erythrocyte by the action of adenylate kinase (*27*).

As to the distribution of P^{32} incorporated into acid-soluble phosphorus compounds in erythrocytes, Table III indicates that 94 per cent of acid-soluble P^{32} was recovered by the chromatography and more than one third was present as ATP. Nearly all of the P^{32} in ATP was in the two labile phosphate groups, between which the equilibrium of specific activity must be reached very rapidly by the action of adenylate kinase in the cells (*27*).

The specific activity of labile P of ADP was almost equal to that of the labile P of ATP, reflecting here again the high activity of adenylate kinase. The specific radioactivity of 2,3-DPGA was considerably lower than that of the cell *i*P as well as of the labile P of ATP and ADP. It is noteworthy that the specific activity of hexose diphosphate and sugar monophosphate fractions was somewhat greater than that of 2,3-DPGA. DPN and TPN fractions were not labeled with P^{32} under the conditions given in this experiment. Atetra P-like nucleotide also took up P^{32} into its labile phosphate groups. AMP was slightly radioactive, probably with the same specific activity as stable phosphate groups in ADP and ATP, though its exact value could not be obtained because of its minute amount. These findings are, at least qualitatively, in confirmation with the observations in our previous studies using paper chromatography (*1*).

DISCUSSION

The results obtained here for human erythrocytes are somewhat different from those presented previously by other workers with the use of differential or paper-chromatographical mothods. Especially, in contrast to most of the former results, the levels of AMP and ADP were found to be very low in the present experiments, the level of AMP being about one hundredth and that of ADP about a tenth of that of ATP when fresh blood sample were analyzed. Aging of the cells seems to cause the increase of AMP and ADP as will be reported elsewhere. In fresh blood cells, ADP originating from ATP is so actively resynthesized to ATP by phosphorylation in glycolytic process that the level of ADP is kept at considerably low level.

That some of the compounds in erythrocytes can be isolated in a high state of purity by column chromatography enables the experimental approach with the use of tracers to obtain more information concerning the metabolism in the cells. Thus the specific activities of labile and stable phosphate groups of ATP and ADP, and of 2,3-DPA could be determined accurately. In confirmation of Gourly (*29*), the uptake of P^{32} by 2,3-DPGA is relatively slow in contrast to labile P of ATP. Gourly proposed a hypothesis that the phosphate enters the cell, forming ATP at the cell surface. It seems, however, to be premature to conclude a precursor relationship merely from the specific activity of the compounds since the cell is not a homogeneous system but must be of more complicated structure. Prankerd and Altman (*30*) reached a different conclusion that 2,3-diphosphoglyceric acid bears a precursor relationship to ATP. An explanation for this discrepancy is that the relatively high incorporation of P^{32} into the ester as observed by these authors would be attributed to the net synthesis of this compound. As has been shown by Guest and Rapoport (*31*), the level of 2,3-DPGA in erythrocyte is by no means constant but varies under different conditions. Thus when the cells are kept in an unfavorable envioronment, such as low temperature or scanty supply of glucose as in the experiments of Prankerd and Altman, the amount of 2,3-DPGA tends to decrease, even if there is little change of the ATP level. When the cells are again placed in a favorable environment, a synthesis of the ester takes place so far as the cells retain viability, and high specific radioactivity of P in 2,3-DPGA may be obtained. Further tracer experiments are being carried out in the authors' laboratory on the chemical changes occurring in the erythrocytes both in fresh state and on storage. The results will be reported later.

SUMMARY

Acid-soluble phosphorus compounds of human erythrocytes were analyzed by column chromatography on Dowex 1-chloride and formate with the use of P^{32} as an indicator. The compound found were uric acid, xanthine, diphosphopyridine nucleotide, adenosine monophosphate, adenosine diphosphate, adenosine triphosphate, inosine monophosphate, phosphoglyceric acid, 2,3-diphosphoglyceric acid, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, and glucose 1,6-diphosphate. An adenine nucleotide with two stable and two labile phosphate groups was also found. The concentrations of these compounds were determined.

After incubation of erythrocytes with P^{32} at 37° for 30 minutes the specific activity of stable P and labile P of ADP and ATP, and of 2,3-diphosphoglyceric acid was estimated with precision. The highest specific acitivity was found in labile P of the nucleotides, and 2,3-diphosphoglyceric acid had considerably lower specific activity. A rapid equilibration of specific activity among labile P of ADP and ATP was accomplished by the activity of adenylated kinase in the cell. There was a slight but distinct incorporation of P^{32} into stable P of ADP and ATP and P of AMP. No P^{32} incorpration was observed in DPN and TPN fractions.

REFERENCES

(*1*) Yoshikawa, H., Nakao, M., Miyamoto, K., and Yanagisawa, I., *J. Biochem.*, **46**, 83 (1959)

(*2*) Bartlett, G. R., Savage, E., and Marlow, H., *J. Appl. Physiol.*, **6**, 51 (1953)

(*3*) Bartlett, G. R., *J. Biol. Chem.*, **234**, 449 (1959)

(*4*) Tatibana, T., and Yoshikawa, H., *Radioisotopes* (*Japan*), **7**, 57 (1958)

(*5*) Cohn, W. E., and Carter, C. E., *J. Am. Chem. Soc.*, **72**, 4273 (1950)

(*6*) Hurlbert, R. B., Schmitz, H., Brumm, A. F., and Potter, V. R., *J. Biol. Chem.*, **209**, 23 (1954)

(*7*) Khym, J. Y., and Cohn, W. E., *J. Am. Chem. Soc.*, **75**, 1153 (1953)

(*8*) Magasanik, B., Vischer, E., Doniger, R., Elson, D., and Chargaff, E., *J. Biol. Chem.*, **186**, 37 (1950)

(*9*) Sekiguchi, T., *J. Japan. Biochem. Soc.*, **26**, 476 (1954)

(*10*) Hanes, O. S., and Isherwood, F. A., *Nature*, **164**, 1107 (1949)

(*11*) Sekiguchi, T., Miyamoto, K., Nakao, M., and Yoshikawa, H., *J. Biochem.*, **45**, 919 (1958)

(*12*) Berenblum, I., and Chain, E., *Biochem. J.*, **32**, 295 (1938)

(*13*) Mejbaum, W., *Z. physiol. Chem.*, **258**, 117 (1939)

(*14*) Roe, J. H., *J. Biol. Chem.*, **107**, 15 (1934)

(*15*) Dische, A., and Borenfreund, E., *J. Biol. Chem.*, **192**, 583 (1951)
(*16*) Dische, A., Shettles, L. B., and Osnos, M., *Arch. Biochem.*, **22**, 169 (1949)
(*17*) Rapoport, S., *Biochem. Z.*, **291**, 429 (1937)
(*18*) Tatibana, M., Nakao, M., Miyamoto, K., Sekiguchi, T., and Yoshikawa, H., *J. Biochem.*, **46**, 711 (1959)
(*19*) Bowen, W. J., and Kerwin, T. D., *J. Biol. Chem.*, **220**, 9 (1956)
(*20*) Horecker, B. L., Ma, T. S., and Haas, E., *J. Biol. Chem.*, **136**, 775 (1937)
(*21*) Newton, E. B., *J. Biol. Chem.*, **120**, 315 (1937)
(*22*) Marrian, D. H., *Biochim. et Biophys. Acta*, **13**, 278 (1954)
(*23*) Lieberman, I., *J. Am. Chem. Soc.*, **77**, 3373 (1955)
(*24*) Dixon, J. S., and Lipkin, D., *Anal. Chem.*, **26**, 1092 (1954)
(*25*) Banderheiden, B. S., Huennekens, F. M., and Gabrio, B. W., *Federation Proc.*, **17**, 327 (1958)
(*26*) Tatibana, M., Nakao, M., Miyamoto, K., Sekiguchi, T., and Yoshikawa, H., *J. Biochem.*, **46**, 711 (1959)
(*27*) Tatibana, M., Nakao, M., and Yoshikawa, H., *J. Biochem.*, **45**, 1037 (1958)
(*28*) Rapoport. S., and Guest, G. M., *J. Biol. Chem.*, **138**, 269 (1941)
(*29*) Gourly, D. R. H., *Arch. Biochem. Biophys.*, **40**, 1 (1052)
(*30*) Prankerd, T. A. J., and Altman, K. I., *Biochem. J.*, **58**, 622 (1954)
(*31*) Guest, G. M., and Rapoport, S., *Physiol. Rev.*, **21**, 410 (1941)

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HEAT DECOMPOSITION PRODUCT OF FLAVIN ADENINE DINUCLEOTIDE IN AQUEOUS SOLUTION

II. PHYSICO-CHEMICAL PROPERTIES OF "FOURTH FLAVIN COMPOUND"

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(Received for publication, November 11, 1959)

In the previous paper of this series (*1*), it was reported that purified FAD was converted to FFC by heating its aqueous solution above 75°, and this new flavin compound was purified by powdered cellulose chromatography and crystallized from its aqueous alcoholic solution. Then, physico-chemical properties of this flavin compound were studied using its crystallized sample, the results are described in this paper.

MATERIALS

FAD—The sample was obtained by our method (*2*) from *Eremothecium ashbyii*. The purity of this sample was above 92 per cent.

FFC—It was prepared from FAD aqueous solution by heating above 100°, purified by powdered cellulose chromatography and crystallized as previously reported (*1*).

FMN and FR—chromatographically purified from commercial samples.

EXPERIMENTAL AND DISCUSSION

Paper Chromatography—As reported preliminarily by one of the authors (*3*), the R_f value of FFC was 0.13 when a mixture of *n*-butanol·acetic acid·water (4 : 1 : 5, *v/v*) was used as a mobile phase. Under the same condition, R_f values of FAD, FMN and FR were 0.03, 0.09 and 0.3 respectively. By using other solvents, the R_f values of FFC were compared with those of other flavins and they were shown in Table I.

Paper Electrophoresis—Paper electrophoresis of FFC and other flavin compounds was practiced by the same method and instrument reported before (*4*). The filter paper (Toyo Roshi, No. 50, 23×5 cm.) had been moistened with a phosphate buffer, and a current of 1.43 mA per cm. of paper-width was applied. Fifteen minutes after that, flavins were placed on the filter paper at the line 8 cm. from the edge of the cathode side. After the application of a current of 1.43 mA per cm. of paper-width for 1.5 hours, flavins

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Abbreviations: FAD, flavin adenine dinucleotide; FFC, fourth flavin compound; FMN, flavin mononucleotide; FR, free riboflavin.

on the paper strip were detected by their fluorescence under ultraviolet light of 365 mμ, and the mobilities were measured.

To examine the second dissociation of phosphoric acid, the mobilities were measured at pH range from 5.0 to 8.0 by using 0.05 *M* phosphate buffer. The results were shown in Table II.

From these data, it was observed that the mobilities of FFC, FAD and FR on the paper did not change with increasing pH. On the contrary, the mobility of FMN was increased above pH 7.0.

These phenomena mean that FMN has a second dissociation of phosphate group, while FFC, FAD and FR have not.

TABLE I

R_f Values of FFC by Various Solvents

Flavins	Solvent				
	1	2	3	4	5
FFC	0.13	0.22	0.03	0.88	0.42
FR	0.30	0.46	0.18	0.40	0.25
FMN	0.09	0.17	0.01	0.85	0.50
FAD	0.03	0.05	0	0.90	0.35

Solvent 1. *n*-Butanol : acetic acid : water, 4 : 1 : 5 (upper layer)
2. *n*-Butanol : pyridine : water, 5 : 3 : 2
3. *n*-Butanol : *n*-propanol : water, 2 : 2 : 1
4. Water saturated with *iso*-amylalcohol
5. 5% Sodium dibasic phosphate aqueous solution

TABLE II

Mobilities of Flavins on Paper

(1.5 hours, 1.43 mA/cm.)

pH	FAD	FFC	FMN	FR
5.0	38	37	38.5	19
6.0	33	31.5	39	15
7.0	33	31	41	14
8.0	33	31	44	14

Absorption Spectrum—Absorption spectrum of FFC in phosphate buffer (0.1 *M*, pH 7.0) was measured by Beckman spectrophotometer. The result is shown in Fig. 1. It was quite similar to that of FMN, and the absorption maxima were found at 223, 267, 373 and 445 mμ which were quite same with those of FMN and FR.

As Whitby (*5*) reported, FMN has a quite similar absorption spectrum with FR above 420 mμ, however, rather lower absorption between 330 and 420 mμ than FR. In every wave length tested, FFC has a quite similar absorption spectrum with FMN, subsequently the chemical structure of FFC is supposed to be similar with that of FMN.

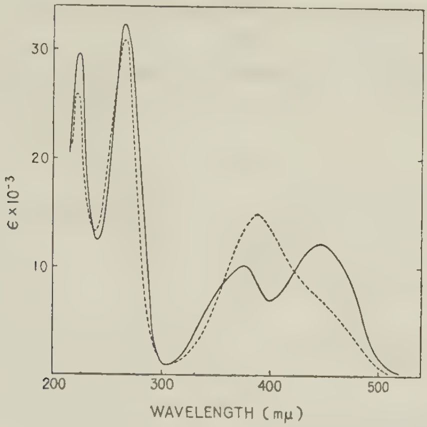

Fig. 1. The absorption spectra of FFC dissolved in 0.1 *M* phosphate buffer at pH 7.0 (solid line), and in *N* HCl (dotted line).

Table III

Molecular Coefficients of FFC, FR, FMN, and FAD

Flavins	pH	260 mμ	265 mμ	375 mμ	450 mμ	References
FFC	7	27.23	31.67	10.34	12.20	Present work
	0	27.60	31.01	13.64	6.76	,,
FR	7	28.10	31.72	10.63	12.20	,,
	7	27.7	—	10.6	12.2	Whitby (*5*)
	0	29.0	31.60	14.05	6.60	Present work
FMN	7	27.16	31.75	10.46	12.20	,,
	7	27.1	—	10.4	12.2	Whitby (*5*)
	0	28.15	31.30	13.56	7.09	Present work
FAD	7	36.84	37.94	9.27	11.31	,,
	7	37.0	—	9.3	11.3	Whitby (*5*)
	0	41.90	42.82	12.81	7.07	Present work

In *N* HCl solution of FFC, the decrease of absorption around 220 mμ and 267 mμ was observed. The absorption maximum at 373 mμ increased and shifted to 388 mμ, and that at 445 mμ decreased to be a small shoulder at 455 mμ. In this case, the yellow color of the solution became a little greenish and the fluorescence of FFC was completely diminished.

The molecular coefficients of FFC at various wave lengths were compared with those of other flavins, as shown in Table III.

Fluorescence of FFC—The fluorescence energy per mole of FFC is quite the same with that of FMN or FR, and the fluorescence energy was not changed after FFC was hydrolysed to FMN or FR.

pH-Fluorescence curves of FFC and FMN were estimated by using buffer of Kavanagh *et al.* (*6*).

The fluorescence intensity was measured by a microphotofluorometer designed by Yagi *et al.* (*7*) (*8*). Ultraviolet ray cut off filter was used, but not fluorescence selective filter.

The pH- fluorescence curve of FFC is similar with that of FMN, as shown in Fig. 2.

The slight difference of pH-fluorescence curve between FFC and FMN was observed in acidic side, *i.e.*, pKa of FMN is existed at 2.17 which is the same with that reported by Bessey *et al.* (*9*), but that of FFC is existed at 1.97. pKa of FFC is also similar with that of FR estimated to be 1.7 by Kavanagh *et al.* (*6*), and 1.97 by Bessey *et al.* (*9*).

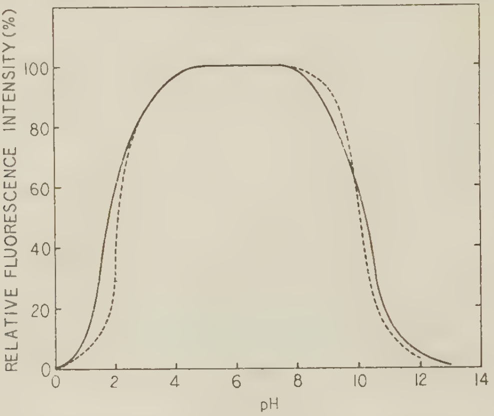

FIG. 2. pH-Fluorescence curves of FFC (solid line) and of FMN (dotted line).

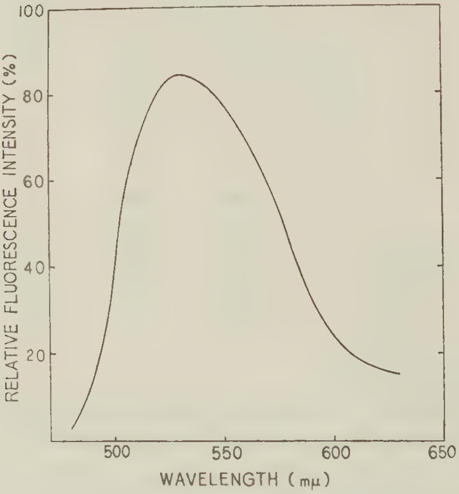

FIG. 3. Fluorescence spectrum of FFC.

Fluorescence spectrum of FFC in an aqueous solution was obtained by fluorospectrophotometer (10), as shown in Fig 3.

The maximum of the fluorescence spectrum is located at 530 mμ as same as those of other flavins, FAD, FMN and FR (*11*).

As reported previously (*3*), FFC was converted to FMN by heating in acetic acid medium, thus considered to be of similar structure with FMN. The present results on the physico-chemical property of FFC also indicate that the structure of FFC is very similar with FMN and that it has not a second dissociation of phosphoric acid.

SUMMARY

FFC can be separated from other flavins, FAD, FMN and FR, by paper chromatography.

Paper electrophoresis at different pH showed that FFC has not a second dissociation of phosphoric acid.

The absorption spectrum of FFC is quite similar with that of FMN, and the maxima of it exist at 267, 373 and 445 mμ, and molecular coefficients are quite similar with those of FMN.

pH-Fluorescence curve of FFC is also similar with that of FMN and the maximum fluorescence energy exists at pH 5.0–7.5, and pKa is 1.97 which is a little smaller than that of FMN, 2.17. The maximum of fluorescence spectrum of FFC is recorded at 530 mμ as same as those of other flavins.

REFERENCES

(*1*) Yagi, K., and Okuda, J., *J. Biochem.*, **47**, 77 (1960)
(*2*) Yagi, K., Matsuoka, Y., Kuyama, S., and Tada, M., *J. Biochem.*, **43**, 93 (1956)
(*3*) Yagi, K., *Kagaku*, **23**, 532 (1953)
(*4*) Yagi, K., and Matsuoka, Y., *J. Biochem.*, **42**, 757 (1955)
(*5*) Whitby, L. G., *Biochem. J.*, **54**, 437 (1953)
(*6*) Kavanagh, F., and Goodwin, R. H., *Arch. Biochem.*, **20**, 315 (1949)
(*7*) Yagi, K., and Arakawa, T., *Kagaku*, **23**, 216 (1953)
(*8*) Yagi, K., and Arakawa, T., *Vitamins*, **6**, 523 (1954)
(*9*) Bessey, O. A., Lowry, O. H., Love, R. H., *J. Biol. Chem.*, **180**, 755 (1949)
(*10*) Yagi, K., Tabata, T., Kotaki, E., and Arakawa, T., *Vitamins*, **7**, 878 (1954)
(*11*) Yagi, K., Tabata, T., Kotaki, E., and Arakawa, T., *Vitamins*, **8**, 61 (1955)

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THE SPECTROPHOTOMETRIC DETERMINATION OF AMINE, AMINO ACID AND PEPTIDE WITH 2,4,6-TRINITROBENZENE 1-SULFONIC ACID*

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The amino acid content of solutions has been most widely determined by the ninhydrin method developed by Moore and Stein (*1*, *2*). Recently, the spectrophotometric method has been also used for an assay of the rate of proteolysis (*5*, *6*) and for a determination of peptide (*3*)- and protein (*4*)-contents in chromatographic effluents. In the latter cases, however, other method seemed to possess more advantages; as the per cent color yield (leucine equivalent (*1*)) showed an appreciable difference from one peptide to another (*6*), and as higher peptide gave a color with ninhydrin in such a low yield that a previous alkaline hydrolysis of a given sample was necessiated (*3*).

In the previous paper, Satake *et al.* (*7*) reported that TNBS reacted with only primary amino group of amino acid and peptide in the aqueous solution at pH 8 and at room temperature without any undesirable side-reactions, and that the resulting TNP-peptide, as well as TNP-amino acid, had a high molar extinction coefficient at 340 mμ. in a similar magnitude. In this paper, we wish to report on the spectrophotometric determination of primary amine, amino acid and peptide by the quantitative TNP-lation with TNBS.

EXPERIMENTALS

Materials—TNBS was prepared from 1-chloro-2,4,6-trinitrobenzene by the treatment with sodium bisulfite in anhydrous methanol at 60°, and purified by the twice recrystallization from *N* HCl after the treatment with active charcoal (*8*). The aqueous solution at a concentration of 1 mg. per ml., was stable in a dark place for at least one week and had an optical density less than 0.05 at 340 mμ.

Authentic TNP-amino acids and -peptides were prepared according to the method previously reported (*7*).

Followings were used as the sample for spectrophotometry: tyramine, histamine, glucosamine, ethanolamine, β-alanine, γ-aminobutyric and *p*-aminobenzoic acids from Wako Junyaku Co. Ltd.; glycine, alanine, valine, leucine, isoleucine, serine, threonine, phenylalanine, tyrosine, tryptophan, cystine, methionine, histidine, lysine, arginine, aspartic and glutamic acids from Ajinomoto Co. Ltd. ; ε-aminocaproic acid prepared from caprolactam; putrescine, cadaverine and isoamylamine prepared by Satake (*12*); glycylglycine, glycylserine, glycylvaline, glycyltyrosine, glycyltryptophan, glycylhistidine, glycylaspartic acid, alanylglycylglycine, leucyltyrosine, alanylaspargine, carnosine and glutathione from the Nutritional Biochemicals Corporation; oxidized insulin A and B obtained from beef insulin by the performic acid-oxidation followed by the fractionation with *M* trichloro acetic acid (*9*).

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* The following abbreviations are used in this paper: TNP, 2,4,6-trinitrophenyl; TNBS, 2,4,6-trinitrobenzene 1-sulfonic acid.

Method—Unless otherwise mentioned, 1 ml. of the sample solution to be analyzed (0.01–0.8 μ mols per ml.), 1 ml. of 4 per cent $NaHCO_3$ and 1 ml. of 0.10 per cent TNBS were mixed and kept in a dark place at 40° for 2 hours. The resulting orange-colored solution (3 ml.) was added with a definite amount of *N* HCl (a ml.) and the optical density of the acidified solution (3+a ml.) was measured at 340 mμ., in the Hitachi spectrophotometer Model EPU-2. One ml. of distilled water, instead of sample solution, was used as a blank.

RESULTS

Absorption Spectrum—When a solution of primary amine, amino acid or peptide in sodium bicarbonate, was added with TNBS, an orange color was produced almost instantly and the color intensity increased during the initial half hour at 40°; while a blank solution, containing no amino compounds, showed only a pale yellow even after several hours. On the acidification,

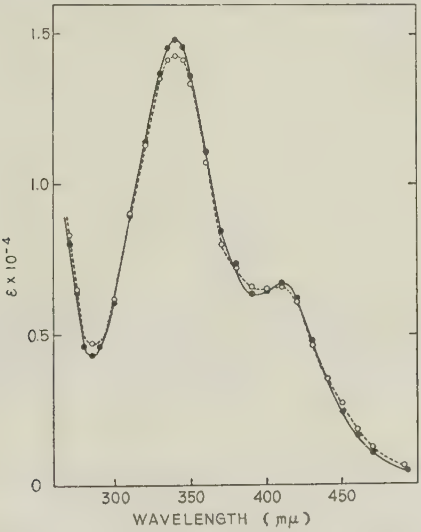


Fig. 1. Absorption spectra of TNP-leucine (—●—) and of a reaction mixture of leucine and TNBS (- -○- -). —●—: 0.030 μ mols per ml. TNP-Leucine (in *N* HCl). - -○- -: 0.126 μ mols per ml. Leucine (1.0 ml.)+4 per cent $NaHCO_3$ (1.0 ml.)+0.1 per cent TNBS (1.0 ml.). After 2 hours at 40°, 1 ml. of *N* HCl was added. All readings were corrected for the corresponding blank values and expressed as the molar extinction coefficient.

the orange color of sample solution changed to yellow which was very stable in a dark place, and the blank solution became almost colorless.

As shown in Fig. 1, the absorption spectrum of the acidified sample solution was practically the same as that of the corresponding authentic TNP-derivative, if the blank values which were only slight in the wave-range longer than 300 mμ., were subtracted from the readings of sample solution. The result indicated that the coloration with TNBS was due to the TNP-lation of primary amino group.

Effect of pH—As shown in Fig. 2, the reaction velocity of primary amino compound with TNBS was markedly influenced by the pH of reaction mixture. Thus the higher the pH, the greater the velocity. In a strong alkaline solution, however, the reaction was accompanied with appreciable amounts of both decompositions of TNBS and of TNP-lated amino compound. These decomposition products had less light absorption in acidic solution than in alkaline at 340 mμ. These results seemed to suggest that the optimum pH for the TNP-lation with TNBS was 7.5 to 8.5 at 40°, and that the spectrophotometry of TNP-lated compound should be done in acidified solution.

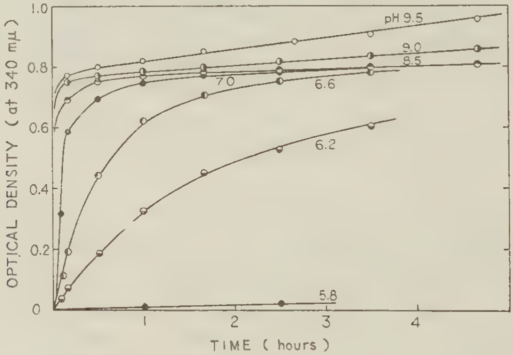


FIG. 2. Effect of pH on the TNP-lation of leucine with TNBS.

One ml. of 0.207 μmols per ml. leucine+1 ml. buffer ($M/10$ KH_2PO_4+ $M/10$ Na_2HPO_4 for pH 5.8–7, $M/10$ KH_2PO_4+$M/20$ $Na_2B_4O_7$ for pH 7–9. and $M/20$ Na_2CO_3+$M/20$ $Na_2B_4O_7$ for pH 9.5)+1 ml. of 0.1 per cent TNBS. After a definite time shown on abscissa at 40°, 1 ml. N HCl was added.

Concentration of TNBS—As shown in Fig. 3, the TNP-lation velocity was proportional to the both concentrations of TNBS and of amino compound to be TNP-lated. So the higher in the concentration of TNBS would be the better, were it not for the fact that the color so obtained was contaminated with too high blank reading for practical purpose. The concentration of TNBS used in this study, 1 mg. per 3 ml. of TNP-lation mixture (approximately 3 μ moles per 3 ml.) was a rather arbitrary one, which seemed to be suitable for the determination of amino acid and peptide in chromatographic effluent (0.01–0.8 μ mols).

Concentration of Amino Compound to be TNP-lated—Fig. 4 illustrates the relationship that exists between optical density and leucine concentration. The result showed that the direct readings of the TNP-lated solution (4 ml.) were just the same as the theoretical values calculated from the molar extiction

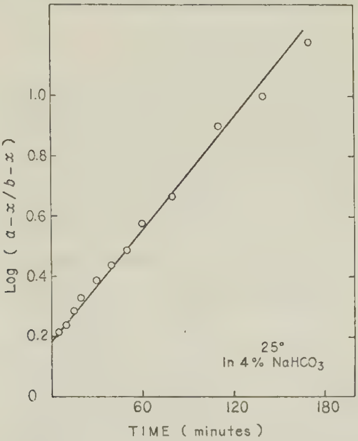


Fig. 3. Reaction velocity of glycine with TNBS at 25°.
a: Concentration of glycine at t=0, 0.612 μ mols per ml. b: Concentration of TNBS at t=0, 0.420 μ mols per ml. x: Concentration of TNP-glycine after *t* minutes, which was calculated from the increase of optical desity at 340 mμ.

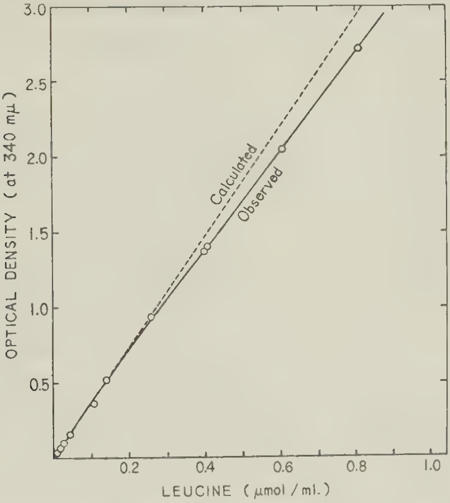


Fig. 4. Relationship between concentration of leucine and optical density after the TNP-lation. One ml. leucine whose concentrations are given on abscissa+1 ml. of 4 per cent $NaHCO_3$+1 ml. of 0.1 per cent TNBS. After 2 hours at 40°, 1.0 ml. *N* HCl was added. Optical densities higher than 0.9, were measured after an appropriate dilution and the readings were multiplied with the dilution factor. All readings were corrected for the corresponding blank values.

TABLE I

Relative Color Intensities Produced from Amines, Amino Acids and Peptides with TNBS (7)

	a (obs.) $\times 10^{-4}$	b (theor.) $\times 10^{-4}$	c (a/b)
Glycine	1.23	1.25	98
Alanine	1.22	1.27	96
Valine	1.34	1.34	100
Leucine	1.42	1.48	96
Isoleucine	1.46		
Serine	1.35	1.42	95
Threonine	1.34	1.33	101
Aspartic acid	1.14	1.15	99
Glutamic acid	1.34	1.41	95
Cystine/2	1.20[1]		
Methionine	1.34	1.34	100
Phenylalanine	1.23		
Tyrosine	1.36	1.35	101
Tryptophan	1.18		
Histidine	1.33	1.35	99
Arginine	1.44		
Lysine/2	1.25[1]		
(mean	1.28)		
β-Alanine	1.23		
γ-Aminobutyric acid	1.26		
ε-Aminocaproic acid	1.17		
p-Aminobenzoic acid	1.36		
(mean	1.25)		
Glycylglycine	1.09	1.10	99
Glycylserine	1.04	1.09	96
Glycylvaline	1.12	1.07	105
Glycylaspartic acid	1.04		
Glycyltyrosine	1.04	1.09	96
Glycyltryptophan	1.01		
Glycylhistidine	1.01		
Alanylglycylglycine	0.98		
Leucyltyrosine	1.10	1.09	101
Alanylaspargine	1.05	1.05	100
Oxidized insulin A	1.03	1.02	101
Oxidized insulin B/	0.99[1]		
(mean	1.05)		
Carnosine	1.34		
Glutathione	1.32		
Histamine	1.36		
Tyramine	1.14		
Glucosamine	1.29		
Putrescine/2	1.26[1]		
Cadaverine/2	1.25[1]		
Isoamylamine	1.30		
Ethanolamine	1.27		
(mean	1.26)		

a) Observed color intensity expressed as the molar extinction coefficient (Just the same method as shown in Fig. 4, was used). b) Molar extinction coefficient reported on authentic TNP-derivative (*7*). c) Per cent color yield, a/b×100.

1) A half of the molar extinction coefficient.

coefficient of authentic TNP-leucine (*7*) up to an optical density of about 0.9 (equivalent to 0.25 μ mols), and that the optical densities of 4 ml. solution calculated from the readings on the further diluted solution (10-30 ml.), was approximately proportional to leucine content up to an optical density of 2.5–3.0 (equivalent to amount 0.75–0.9 μ mols.).

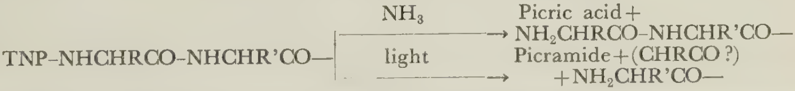
Relative Color Intensities of Various Amines, Amino Acids and Peptides—In Table I, the relative color intensities of primary amines, amino acids and peptides are shown as the molar extinction coefficients which were calculated from the optical density at 340 mμ. and from the concentration of sample in the final diluent, together with those of the authentic TNP-compounds (*7*).

DISCUSSION

From the above-mentioned results, it was concluded that amino acids, peptides and primary amines could be assayed spectrophotometrically by the TNP-lation with TNBS, using the following conditions: 1.0 ml. of sample solution to be analyzed (at a concentration of 0.01–0.8 μ mols per ml.), 1.0 ml. of 4 per cent sodium bicarbonate and 1.0 ml. of 0.1 per cent TNBS were mixed, kept in a dark place at 40° for 2 hours and the optical density was measured at 340 mμ., after the acidification with a definite volume of *N* hydrochloric acid.

The readings so measured were practically just the same as that of the equivalent amount of the corresponding TNP-derivative, thus about 1.27 $(1.14\text{–}1.46)\times 10^4$ for amino groups of amines and amino acids and 1.05 $(0.98\text{–}1.12)\times 10^4$ for those of peptides at a final concentration of *N*, respectively. The high values (1.3×10^4) of two peptides, carnosine and glutathione, may be due to their exceptional structure. The similiarity of the relative color intensities among various peptides, from simple dipeptide to oxidized insulin, will make this spectrophotometry to be valuable for an assay of peptide. We have used this method, with complete satisfaction for the determination of peptide content in chromatographic effluents* and for an assay of the rate of proteolysis (*10*, *11*).

In connection with the former analysis, it should be emphasized that each peptide could be recovered with simple procedures (*7*) as the TNP-derivative after the assay, and that the TNP-peptides so obtained, could be used for the N-terminal and non-N-terminal amino acids analysis by the usual methods (*7*). According to Sasakawa*, α-TNP-group could be removed as picric acid with concentrated ammonia so easily that TNP-peptide regenerate the corresponding peptide without any appreciable splitting of the peptide bonds. As already reported by the present authors (*13*), TNP-peptide could be also decomposed with light as follow:



$$\text{TNP-NHCHRCO-NHCHR'CO—}\left|\begin{array}{l} \xrightarrow{\quad NH_3 \quad} \text{Picric acid} + NH_2CHRCO\text{-}NHCHR'CO\text{—} \\ \xrightarrow{\quad \text{light} \quad} \text{Picramide} + (CHRCO\,?) + NH_2CHR'CO\text{—} \end{array}\right.$$

The combination of these interesting reactions with this spectrophotometry will be useful for peptide analysis.

SUMMARY

1. Primary amine, amino acid, and peptide at a concentration of 0.01 to 0.8 μ mols per ml., could be assayed spectrophotometrically by the TNP-lation with TNBS.

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* Unpublished data (Sasakawa, S.).

2. Under the conditions described, the optical densities of various amino compounds were practically the same as those of the equivalent amount of the corresponding TNP-derivatives, which were 1.27×10^4 for amino groups of amines and amino acids and 1.05×10^4 for those of peptides at a final concentration of N, respectively.

3. TNP-lated sample, after the assay, could be recovered from the spectrophotometric mixture and be used for the structural analysis.

We are indebted to Mrs. S. Sasakawa for the information on her unpublished data and to Mr. J. Nitta for the preparation of TNBS. This work was supported in part by the grant of Scientific Research Found of the Ministry of Education for which the authors wish to thank.

REFERENCES

(*1*) Moore, S., and Stein, W. H., *J. Biol. Chem.*, **176**, 367 (1948)

(*2*) Spackman, D. H., Stein, W. H., and Moore, S., *Anal. Chem.*, **30**, 1190 (1958)

(*3*) Hirs, C. H. W., Moore, S., and Stein, W. H., *J. Biol. Chem.*, **219**, 623 (1956)

(*4*) Crampton, C. F., Moore, S., and Stein, W. H., *J. Biol. Chem.*, **215**, 787 (1955)

(*5*) Crampton, C. F., Stein, W. H., and Moore, S., *J. Biol. Chem.*, **225**, 363 (1957)

(*6*) Izumiya, N., and Uchino, H., *J. Biochem.*, **46**, 235 (1959)

(*7*) Okuyama, T., and Satake, K., *J. Biochem.*, **47**, 454 (1960)

(*8*) Satake, K., and Okuyama, T., 'Electrospectrophotometry in Biological Chemistry' ed. by 'Kagaku-no-Ryoiki', Nankôdô, Tokyo, Vol. **2**, p. 63 (1958)

(*9*) Yamamoto, M., Satake, K., Okuyama, T., and Kotaki, A., Symposium on Protein Chemistry held at Osaka, on Oct. 31st, 1959. Abstract p. 47

(*10*) Sasakawa, S., and Satake, K., *J. Biochem.*, **45**, 867 (1958), **47**, 672 (1960)

(*11*) Satake, K., and Luck, J. M., Federation Proc., **18**, 313 (1959)

(*12*) Satake, K., Ando, S., and Fujita, H., *J. Biochem.*, **40**, 299 (1953)

(*13*) Satake, K., and Okuyama, T., *Bull. Chem. Soc. Japan*, **32**, 526 (1959)

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PHOSPHORUS METABOLISM IN HUMAN ERYTHROCYTE

III. REGENERATION OF ADENOSINE TRIPHOSPHATE IN LONG-STORED ERYTHROCYTE BY INCUBATION WITH INOSINE AND ADENINE*

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It has been observed by several workers that the level of ATP in erythrocytes gradually declines during storage of the blood in acid-citrate-dextrose solution (ACD) at 4°. Gabrio *et al.* (*1*) proposed from this fact that the decrease of post-transfusional viability of the cells is attributed to the diminished concentration of ATP after the period of preservation. The ATP level of stord blood is restored to some extent by incubation with adenosine or inosine and the ability of these cells to survive in the circulation after transfusion is also improved (*1–3*). When the period of storage is prolonged to more than several weeks, ATP in the cells is not regenerated any longer by the addition of inosine alone. This may be accounted for by the depletion of the adenine moiety needed for the resynthesis of ATP inside the erythrocytes. The expectation that ATP level may be restored if this system is supplimented with adenine has been realized as reported in a preliminary communication (*4*). The present paper describes in detail the experiments on this subject.

MATERIALS AND METHODS

Human blood was collected in ACD medium (1.33 g. of glucose, 1.32 g. of sodium citrate and 0.48 g. of citric acid in 100 ml.) and stored in a refrigerator at 4°. The ratio of the volume of blood to ACD solution was 4 : 1 in all instances.

Inosine, adenosine, guanosine, thymidine, cytidine, uridine, adenine, guanine, uracil,

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* This work was aided by a grant from the Scientific Research Fund of the Ministry of Education.

Abbreviations as follows: AMP, adenosine 5′-monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; AtetraP, adenosine tetraphosphate; IMP, inosine 5′-monophosphate; DPN, diphosphopyridine nucleotide; PGA, phosphoglyceric acid; DPGA, diphosphoglyceric acid; SMP, sugar monophosphate; HDP, hexose diphosphate; *i*P, inorganic orthophosphate.

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thymine, xanthine and orotic acid were obtained from Sigma Chemical Company and Nutritional Biochemicals Corporation; deoxyadenosine and deoxyguanosine from California Foundation for Biochemical Research, Inc.; adenine-8-C^{14} from Schwarz Laboratories Inc.

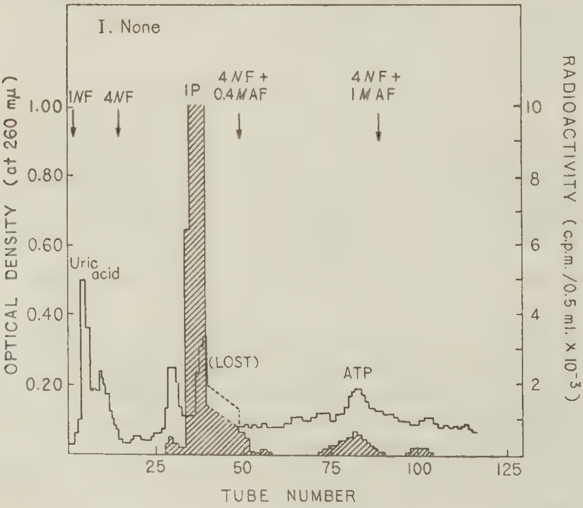
In most of the experiments the acid-soluble phosphorus compounds were analyzed by column chromatography with Dowex 1-formate (*5*) as employed for blood in a previous paper (*6*). Total phosphorus was determined by the method of Horecker *et al.* (*7*) with slight modification by Sekiguchi (*8*), true iP and labile phosphate group according to the method of Martin and Doty (*9*), and ribose by orcinol method (*10*). The radioactivity of P^{32} was measured with a conventional end-window Geiger-Müller counter (Scientific Research Institute, Tokyo).

Estimation of P^{32} incorporation into nucleotides was performed as follows: One milliliter of blood sample incubated with P^{32} was extracted with 6 ml. of ice-cold 0.6 N perchloric acid (PCA). To 5 ml. of the extract was added about 200 mg. of Norit SX 30, which had been previously activated by heating and treated with 8 per cent octanol in ethanol (*11*). After centrifugation, the supernatant was discarded and the charcoal which adsorbed the nucleotides nearly completely from the extract was washed three times with cold water containing a small amount of carrier iP. Then, the charcoal was transferred to a sample dish with the aid of water, dried under a heater lamp and counted with a Geiger-Müller tube.

RESULTS

Chromatography of Acid-Soluble Fraction—An experiment was designed to study the regenration of ATP when long-stored blood was supplimented with inosine and adenine. A blood specimen which had been stored for 8 weeks was divided into three portions and each was incubated at 37° for 2 hours with or without addition of adenine and inosine.

Fig. 1 presents the results which were obtained by ion exchange resin chromatography of perchloric acid (PCA) extracts of the three portions. The elution curves are plotted from the adenine and P^{32} values.



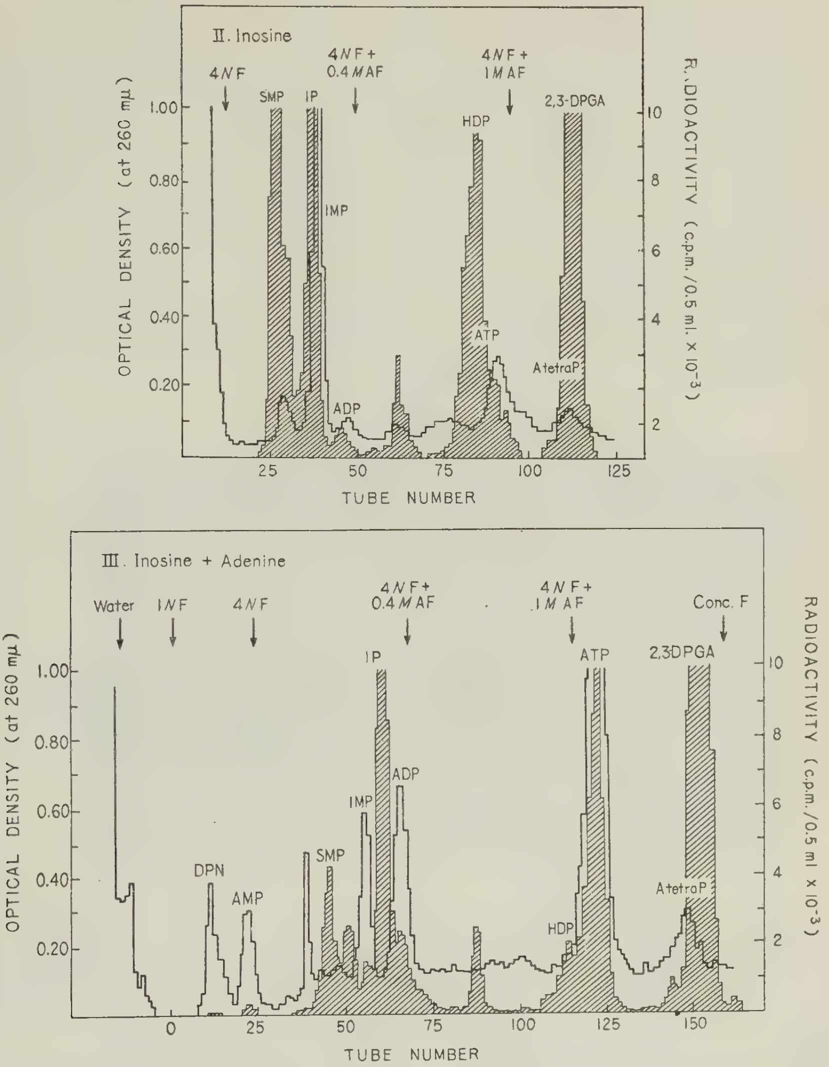


Fig. 1. Chromatography of the acid-soluble phosphorus compounds of human blood (stored 8 weeks) incubated with P^{32} with and without addition of inosine alone and inosine plus adenine. An ACD blood specimen stored for 8 weeks was divided into three 50 ml. portions and each was incubated at 37° for 3 hours with addition of one tenth volume of isotonic NaCl containing 40 to 125 μc. P^{32} and following suppliments per ml.: I, none; II, 125 μmoles inosine; III, 175 μmoles inosine and 100 μmoles adenine. After incubation, the erythrocytes were washed, extracted with PCA and the neutralized extract was submitted to chromatography on a 1 cm^2. × 15 cm. column of Dowex 1-X10 formate employing gradient elution technique. The mixing flask contained 300 ml. of water initially and the reservoir was changed to 1 *N* formic acid (F), 4 *N* F, 4 *N* F and 0.4 *M* ammonium formate (AF), and 4 *N* F and 1 *M* AF at the arrows. Fractions of 10 ml. were collected. Optical density at 260 mμ. is represented by empty and radioactivity by shaded columns.

The peak fractions (from No. 116 to 130) of the elution range corresponding to ATP from the blood sample incubated with added inosine and adenine gave constant ratios of E_{260} to E_{275} and of P^{32} to E_{260}. Furthermore, the fraction of collection tube No. 126 gave a molar ratio, 1 : 0.97 : 1.95 : 3.12, of adenine to ribose to labile phosphorus to total phosphorus. The pooled fractions were rechromatographed on Norit SX 30 with the use of 0.2 *N* NH_4OH in 30 per cent ethanol for elution. The eluate gave a typical spectrum of adenine nucleotide with a maximum at 258 mμ. both in alkali and acid. These findings indicate that the material eluted in the peak fractions is composed of almost exclusively ATP.

In Table I are summarized the assay values of the phosphate esters obtained from the chromatograms illustrated in Fig. 1.

Table I

Changes of Phosphorus Compounds on Incubation with Inosine and Adenine of Blood Which Had Been Preserved 8 Weeks

The same experiment as given in Fig. 1. All values are expressed as μmoles per 100 ml. ACD Blood.

Phosphorus compound	I	II	III
AMP	—	—	1.9
IMP	4.7	15	6.8
ADP	—	0.4	7.2
ATP	1.5	2.5	27.4
AtetraP like compound	0.25	0.45	2.6
2, 3-DPGA	1.5	74	43.5
HDP fraction[1)]	20.5	44	7.0
SMP fraction[1)]	8.5	70	3.5

1) Expressed as P.

In the samples containing added inosine but not adnnine, 2,3-DPGA was increased considerably, whereas the increase in ATP was not significant. In the blood incubated with both inosine and adenine the ATP level was increased up to more than 70 per cent of that in fresh blood (*6*).

Incorporation of Adenine-8-C^{14} into Regenerated ATP—To 5 ml. of ACD blood which had been preserved for 10 weeks were added 20 μc. of P^{32}, 10 μc. of adenine-8-C^{14} and 10 μmoles of inosine, and the mixture was incubated for 3 hours at 37°. After incubation, PCA extract was prepared from the washed erythrocytes and passed through a column of a mixture of 200 mg. of Norit SX 30 treated previously with octanol as described above and of 200 mg. of Hyflo Supercel. The charcoal on which nucleotides had been absorbed was washed with several ml. of water, and then eluted with 0.2 *N* NH_4OH in 30 per cent ethanol. The effluent was condensed under a reduced pressure at below 30° and an aliquot subjected to paper chromatography by ascending method run in *n*-butyric acid-ammonia (20 : 12) (*12*). The chromatogram was radioautographed immediately after and about a hundred days after the separation with the use of two sheets of X ray film (Fuji No-Screen Type 200) put one over the other. As seen in Fig. 2, there appeared blackening corresponding to the spots of ATP, ADP and AMP on both of the sheets of film when the radioautography was made immediately after the paper chromatographic separation of the nucleotides. This blackening is due to high energy beta ray from P^{32} taken up by the adenine nucleotides. On the other hand, when the radioautograph was prepared after P^{32} had decayed off nearly completely, only the sheet of film which had been contacted directly to the paper chromatogram gave the corresponding image while the other remained transparent, because the beta ray emmited from C^{14} is so soft that it does not reach the second film. This means that the added adenine was utlized for the synthesis of adenine nucleotides together with inorganic phosphate.

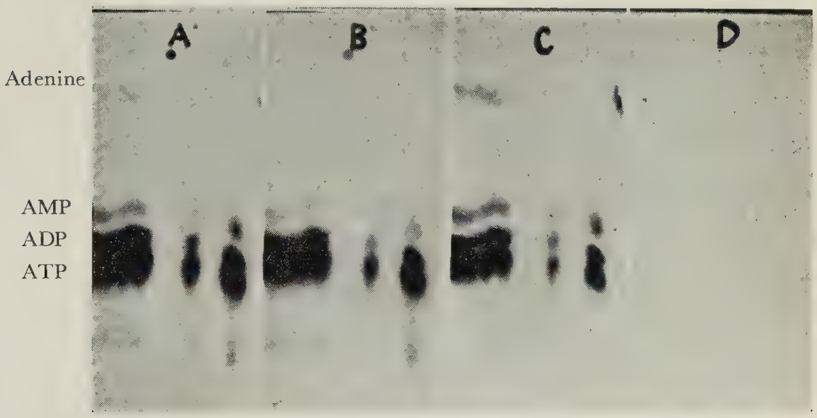


FIG. 2. Paper chromatographic separation of doubly labeled AMP, ADP and ATP synthesized in aged blood cells by incubation with added P^{32}, adenine-C^{14} and inosine. A and B, immediately after the separation, 2 day exposure; C and D after 100 days, 7 day exposure; A and C, the film directly faced to the chromatogram; B and D, the film screened by the former.

Shape Transformation—It has been recognized generally that erythrocytes become spheric when preserved for over several weeks in the cold. On incubation with added inosine and adenine, when ATP was regenerated inside the cells, almost all of the erythrocytes were transformed to a shallow bowl form, being concave on one side as previously reported briefly (*4*). This phenomenon began to appear after 30 minute incubation and was complete within 2 hours (Fig. 3).

Influence of Inosine and Adenine Concentrations on Incorporation of P^{32} into Nucleotides—The extent of ATP regeneration can be estimated by measuring P^{32} incorporation into total nucleotide of erythrocytes since the major component of the nucleotide which is synthesized during incubation with added inosine

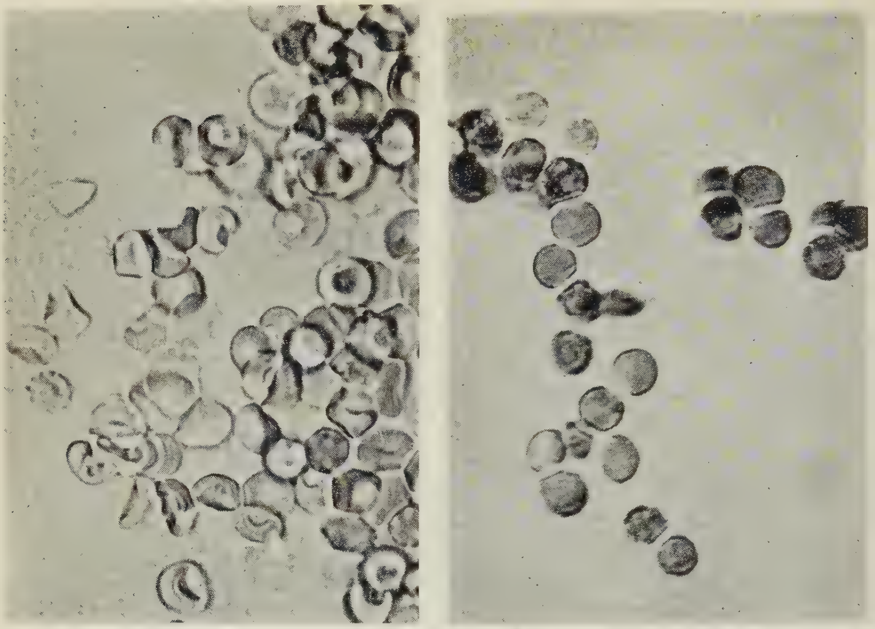


FIG. 3. Microphotographs of erythrocytes which had been stored 8 weeks and were then incubated 3 hours at 37° with (A) and without (B) addition of inosine and adenine. The cells were agglutinated by isohemagglutinin.

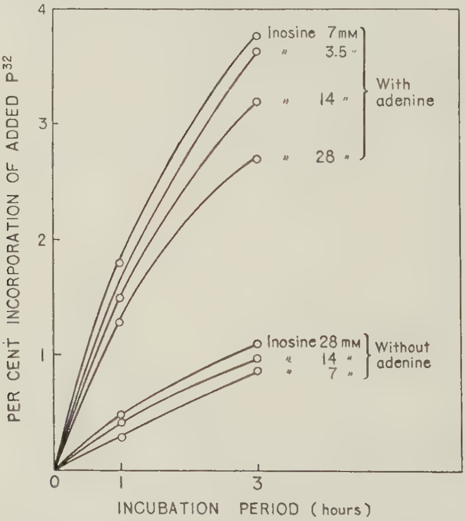


FIG. 4. Influence of inosine concentration on P^{32} incorporation into nucleotides. The reaction mixture contained 1 ml. of ACD blood which had been stored for 9 weeks, 0.1 ml. of P^{32} in physiological saline with the same phosphate concentration as in serum and 0.1 ml. of the solution containing an amount of adenine to make the final concentration 2×10^{-3} *M* and varying amounts of inosine. Incubation was carried out at 37°. The radioactivity of nucleotide absorbed on Norit SX30 was measured.

and adenine has been indentified as ATP from the foregoing experiments. On the basis of this consideration, P^{32} incorporation into total nucleotide was studied under various concentration of added inosine and adenine.

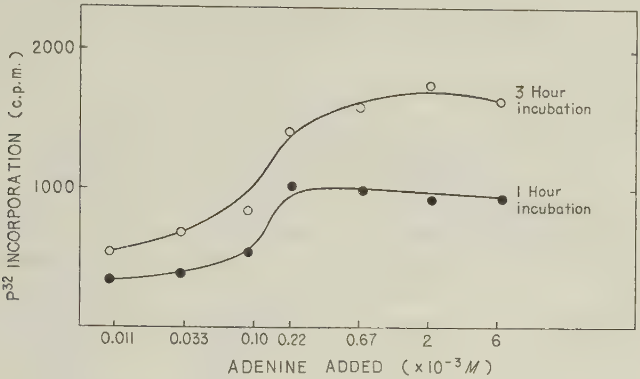


FIG. 5. Influence of adenine concentration on P^{32} incorporation into nucleotides. Experimental conditions were as for Fig. 4 except that the concentration of inosine was fixed at 3.5×10^{-3} *M* and the amount of added adenine was varied.

Fig. 4 presents the results which were obtained by varying the concentration of inosine, and Fig. 5 by varying the concentration of adenine. Although the results show that a wide range of concentration of inosine as well as of adenine added is effective for P^{32} incoroporation into nucleotides, a rather lower concentration of inosine such as 3.5×10^{-3} *M* (final concentration) seems to be more effective than higher cencentrations and adenine develops its maximal effect at the concentration more than 0.67×10^{-3} *M*. Accordingly, in most of further experiments the final concentrations of added inosine and adenine were adjusted to 3.5×10^{-3} *M* and 2×10^{-3} *M*, respectively.

Influence of Preserved Period—The results obtained from the experiments in which ACD blood preserved for various period were incubated with and without supplimentation of adenine and inosine were summarized in Table II. In fresh blood, a rapid incorporation of P^{32} into nucleotide was observed, reflecting an active turnover of labile phosphate groups of ATP and ADP. The effect of addition of inosine or both inosine and adenine was not distinct. As the preservation period was prolonged, the rate of P^{32} incorporation dropped markedly in 16 days when the blood was not suplimented with inosine or inosine plus adenine, while addition of inosine alone or inosine plus adenine prevented the decrease of P^{32} incorporation rate considerably. After storage of much longer periods, the difference between supplimentation with inosine alone and with both inosine and adenin became greater.

Influence of Metabolic Inhibitors—Table III summarizes experiments on the effects of some metabolic inhibitors. Fluoride inhibited the rate of P^{32} incorporation into nucleotides 87 per cent at 1×10^{-2} *M* and 30 per cent at

$1\times10^{-3}\,M$, and arsenate 90 per cent at $1\times10^{-2}\,M$ and 22 per cent at $1\times10^{-3}\,M$. The most effective inhibitor was monoiodoacetate which inhibited completely the regeneration of nucleotides at the concentraction as low as $1\times10^{-3}\,M$. Moreover, it is noteworthy that there exists a conspicuous parallelism between inhibitory effects on nucleotide resynthesis and on shape transformation of the cells.

TABLE II

Influence of Preservation Period of ACD Blood on Rate of P^{32} Incorporation into Nucleotides in Erythrocytes

Experimental details were the same as for Fig. 4. Incubation time, 3 hours; final concentrations of added inosine and adenine, $3.5\times10^{-3}\,M$ and $2\times10^{-3}\,M$, respectively. The values represent per cent incorporation of total added P^{32}.

Preservation period	Control	Inosine alone	Inosine and adenine
3 hours	9.2	12.6	12.7
16 days	2.9	6.0	7.5
36 days	0.5	1.2	3.1
45 ,,	0.5	2.3	5.6
56 ,,	0.5	0.8	3.2
67 ,,	<0.1	—	2.9

TABLE III

Influence of Metabolic Inhibitors on P^{32} Incorporation into Nucleotides in Erythrocytes

The experimental conditions were as for Table II. Total radioactivity of added P^{32} was 69,360 c.p.m. Inhibitors were added in the form of sodium salt.

Adenine	Inosine	Inhibitor, concentration in mixture		P^{32} incorporated c.p.m.	Shape change
—	—	None		164	—
+	+	None		3706	+
+	+	Arsenate	$(1\times10^{-3}M)$	2893	+
+	+	,,	$(1\times10^{-2}M)$	398	—
+	+	Iodoacetate	$(1\times10^{-3}M)$	63	—
+	+	,,	$(1\times10^{-2}M)$	108	—
+	+	Fluoride	$(1\times10^{-3}M)$	2585	+
+	+	,,	$(1\times10^{-2}M)$	501	—

Specificity of Added Nucleoside and Base—Substitution of inosine and adenine with various other nucleosides and bases gave results presented in Table IV.

Guanine, xanthine, uracil, thymine and orotic acid exerted no stimulating effect on P^{32} incorporation into mucleotides of erythrocytes when added in place of adenine to long-stored blood supplimented with inosine. This indicates that only adenine nucleotides are synthesized in the cells of the blood. When nucleosides were added to the stored blood supplimented with adenine, purine nucleosides such as inosine, adenosine, guanosine, deoxyadenosine and deoxyguanosine were effective whereas pyrimidine nucleosides such as uridine, cytidine and thymine were without, if any, effect on the regeneration of the nucleotides. It is of interest that the effective nucleosides, inosine and guanosine, can be split by nucleoside phosphorylase purified from erythrocyte by Gabrio and Huennekens (*13*). It is clear that in confirmation of several authors (*14–16*) nucleosides undergo phosphorolysis in the cell with subsequent metabolism of the ribose moiety to provide the pentose residue of the adenine nucleotides and the glycolytic intermediates necessary for the supply of phosphate. Adenosine which is not attacked by the enzyme must be deaminated in the cell by the presece of active adenosine deaminase (*17*) to inosine first and utilized. The data presented in the table also suggest that purine deoxynucleosides can be metabolized similarly by erythrocytes. It is to be recalled that nucleoside phosphorylase obtained from liver is active with both ribonucleosides and deoxyribonucleosides (*18*, *19*). A slight but distinct increase of P^{32} incorporation by incubation with added adenosine alone may be accounted for by the presence of a small

TABLE IV

Effect of Various Nucleosides and Bases on P^{32} Incorporation into Nucleotides in Erythrocytes

The reaction mixture contained 1 ml. of ACD blood stored for 10 weeks, 68,400 c.p.m. of P^{32}, 1 mg. of nucleoside and 0.5 mg. of base. Incubation, 3 hours at 37°.

Nucleoside added	Base added	P^{32} incorporated c.p.m.	Shape change
None	None	75	–
Adenosine	,,	1316	±
Guanosine	,,	911	–
Inosine	Adenine	1982	+
Adenosine	,,	2232	+
Guanosine	,,	2235	+
Uridine	,,	90	–
Cytidine	,,	98	–
Deoxyadenosine	,,	2404	+
Deoxyguanosine	,,	2508	+
Thymidine	,,	243	–
Inosine	Guanine	424	–
,,	Xanthine	354	–
,,	Uracil	415	–
,,	Thymine	415	–
,,	Orotic acid	336	–

amount of adenine as a contaminant or by the occurrence of another enzymic reaction within the intact cells to liberate adenine.

It is obvious from Table IV that, here again, the shape transformation from spheric to shallow bowl form takes place in intimate relation to the resynthesis of the nucleotides in aged cells.

DISCUSSION

A previous observation (*3*) indicates that it is necessary to reserve a sufficient amount of adenine moiety inside the preserved erythrocytes for the restoration of ATP by incubation with added nucleoside such as inosine. Upon prolonged storage there is a marked decline in the level of total adenine nucleotide in the cells. Attempts to supply adenine moiety by adding ATP, ADP, AMP or ribose 5-phosphate and adenine to the blood failed as demonstrated by Gabrio *et al.* (*1*), since such phosphorylated substances do not readily penetrate the erythrocyte membrane. Adenosine does enter the cell but appears to be utilized for ATP formation only after the nucleoside has deaminated within the cells. Thus, added adenosine does not, if any, serve as source of adenine moiety but supplies ribose phosphate as a substrate for energy metabolism just as inosine does.

There exists little or no activity of adenine deaminase in human erythrocytes in contrast to its presence in avian erythrocytes. Our evidence indicates that added adenine enters the cell interior and is itself used to build up ATP upon incubation of stored blood together with added inosine, even when the blood has been preserved so long that the total amount of adenine nucleotide has declined to exceedingly low level. These findings lead us to a prospect for extending the useful storage life blood cells and possibly of other tissue cells. The shape change of aged cells induced by incubation with supplimented inosine and adenine might be regarded as an evidence of the improvement of cellular fuctions. Adenosine triphosphate seems to play a role in maintaining the disk shape of the red cell, presumably by causing a transformation of the protein of the erythrocyte stroma. Further studies on this phenomenon will be reported elsewhere.

SUMMARY

1. ATP content of long-stored blood cells was not restored by incubation with inosine alone but a marked increase of ATP level was observed by incubation with added adenine together with inosine.

2. By the use of adenine-8-C^{14}, it was demonstrated that added adenine itself was utilized for the synthesis of adenine nucleotides.

3. Regeneration of ATP was accompanied by a shape change of erythrocytes from spheric to shallow bowl form.

4. The extent of ATP regeneration in aged erythrocytes can be estimated by measuring P^{32} incorporation into total nucleotide in the cells. By this procedure was studied the influences of adenine and inosine concentrations,

preservation period, and metabolic inhibitors. Arsenate, iodoacetate and fluoride inhibited the reaction strongly.

5. Only adenine was effective among various purines and pyrimidines. When various nucleosides and deoxynucleosides were added to the stored blood supplimented with adenine, purine nucleosides and purine deoxynucleosides were effective. The nucleosides and possibly the deoxynucleosides are suggested to provide the pentose residue of the adenine nucleotides and the glycolytic intermediates necessary for the supply of phosphate.

6. These findings lead us to a prospect for extending the useful storage life of aged blood cells.

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REFERENCES

(*1*) Gabrio, B. W., Hennesey, M., Thomasson, J., and Finch, C. A., *J. Biol. Chem.*, **215**, 357 (1955)

(*2*) Gabrio, B. W., Finch, C. A., and Huennekens, F. M., *Blood*, **11**, 103 (1956)

(*3*) Prankerd, T. A. J., *Biochem. J.*, **64**, 209 (1956)

(*4*) Nakao, M., Nakao, T., Tatibana, M., Yoshikawa, H., and Abe, T., *Biochim. et Biophys. Acta*, **32**, 564 (1959)

(*5*) Hurlbert, R. B., Schmitz, R. B., Brumm, A. F., and Potter, V. R., *J. Biol. Chem.*, **209**, 23 (1954)

(*6*) Yoshikawa, H., Nakao, M., Miyamoto, K., and Tatibana, M., *J. Biochem.*, **47**, 635 (1960)

(*7*) Horecker, B. L., Ma, T. S., and Haas, E., *J. Bol. Chem.*, **136**, 775 (1937)

(*8*) Sekiguchi, T., *J. Japan. Biochem. Soc.*, **26**, 476 (1954)

(*9*) Martin, J. B., and Doty, D. M., *Anal. Chem.*, **21**, 965 (1949)

(*10*) Mejbaum, W., *Z. physiol Chem.*, **258**, 117 (1939)

(*11*) Threlfall, C. J., *Biochem. J.*, **65**, 694 (1957)

(*12*) Ohmura, K., and Fukushi, S., *J. Agr. Chem. Soc. Japan*, **27**, 515 (1953)

(*13*) Gabrio, B. W., and Huennekens, F. M., *Biochim. et Biophys. Acta*, **18**, 585 (1955)

(*14*) Rubinstein, D., Kashket, S., and Denstedt, O. F., *Can. J. Biochem. Physiol.*, **34**, 61 (1956)

(*15*) Gabrio, B. W., Donohue, D. M., Huennekens, F. M., and Finch, C. A., *J. Clin. Invest.*, **35**, 657 (1956)

(*16*) Kawachi, T., and Yoshizumi, S., *J. Japan. Biochem. Soc.*, **29**, 681 (1957)

(*17*) Conway, E. J., and Cook, R. B., *Biochem. J.*, **33**, 457 (1939)

(*18*) Friedkin, M., and Kalckar, H. M., *J. Biol. Chem.*, **184**, 437 (1950)

(*19*) Manson, L. A., and Lampen, J. O., *J. Biol. Chem.*, **191**, 95 (1951)

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STUDIES ON HEMOGLOBIN

IV. HISTIDINE PEPTIDES DERIVED FROM HORSE GLOBIN WITH STREPTOMYCES GRISEUS PROTEINASE*

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During the studies on arginine peptides derived from hemoglobin (*1*), histone (*2*), and protamine** with *Streptomyces griseus* proteinase (*3*), it was elucidated that the proteinase was capable to hydrolyse a wide range of peptide bonds but with a remarkable substrate specificity on the hydrolysis of -X-Arg- linkages. In this paper, we wish to report the results on the analysis of histidine peptides derived from horse hemoglobin with this proteinase.

Histidine peptides in the enzymic hydrolyzate, were separated on a column of Amberlite IRC-50 at pH 5.2 (*5*) and purified as the α-mono-TNP-histidine peptides. From the acid hydrolyzate of the TNP derivative, α-mono-TNP-histidine on the N-terminal and free histidine on the non-N-terminal positions could be recovered in good yield, respectively (*4*).

EXPERIMENTALS

Materials—Horse globin and *Streptomyces griseus* proteinase preparation (*3*) used in this experiment, were the same ones as those used in the previous paper (*1*). 2,4,6-Trinitrobenzene 1-sulfonic acid, 1-chloro-2,4,6-trinitrobenzene and authentic α-mono-TNP-amino acids were obtained by the method of Satake *et al.* (*4*).

Enzymic Hydrolysis of Globin—To a suspension of 1 g. of heat-denatured globin in 20 ml. of $M/100$ trisodium citrate, was added 20 mg. of *Streptomyces griseus* proteinase as 1 per cent solution in the same buffer. The suspension, after the pH was adjusted to 7.5, was kept at 37° for a definite time. The rate of the hydrolysis was calculated from the free amino contents of the enzymic and complete (6 N HCl, 110°, for 20 hours) hydrolyzates which were assayed spectrophotometrically, according to TNP-method (*6*).

Chromatographic Separation of Histidine Peptides—Enzymic hydrolyzate corresponding to 250 to 350 mg. of globin, was concentrated in a rotary evaporator to a few ml., and added to a column of Amberlite IRC-50 (1.8 × 140 cm., 150-200 mesh) which had been previously equilibrated with $M/2$ sodium citrate buffer (Na 1 g. ion per liter, pH 5.2) (*5*).

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[*] The following abbreviations are used: TNP, 2,4,6-trinitrophenyl; O. D.$^{\mathrm{TNP}}$, optical density at 350 mμ of TNP derivative in 4 per cent $NaHCO_3$ (*4*); O. D.$^{\mathrm{Im}}$, opticol density of color produced by histidine peptide with diazobenzene sulfonic acid (*8*).

[**] Unpublished data (Satake, K)

The basic peptides absorbed on the column, were eluted with the same buffer and finally with *N* sodium hydroxide, at a rate of 9±1 ml. per hour. The effluent was collected in 5 ml.-fractions.

The concentration of histidine peptides was detremined spectrophotometrically with diazotized sulfanilic acid (O. D.$^{\mathrm{Im}}$) (*8*), assuming the color intensities were just the same as that of equivalent free histidine.

The concentration of tyrosine, which was also diazoreaction positive, was determined with 1-nitroso-2-naphthol (*7*).

Purification of Histidine Peptides—The effluent fractions corresponding to each diazo-reaction positive but nitrosonaphthol reaction negative peak, were separately passed through a column of Amberlite IRA-411 (OH-phase, 3×15 cm., 50-100 mesh) and washed with 200 ml. of water (*1*, *2*) then with 150 ml. of 2 *N* ammonia, at an approximate rate of 1 ml. per minute. All diazo-reaction positive components still remained on the column, were finally eluted with 100 ml. of 10 per cent acetic acid, together with small amount of non-histidine peptides which could be removed as the ether-soluble TNP-derivatives during the following procedure.

The acetic acid-eluate, after concentration to dryness below 50°, was dissolved in 5 ml. of *M*/2 Na_2HPO_4 (the final pH should be approximately 8 (*4*, *6*)). The solution was added with 5 ml. of 0.5 per cent aqueous solution of 2,4,6-trinitrobenzene 1-sulfonic acid (or 0.5 per cent solution of 1-chloro-2,4,6-trinitrobenzene in ethanol) and the mixture was kept in a dark place at room temperature for 5 hours (*4*). The TNP-lated mixture was then acidified to pH 1 and washed with peroxide-free ether repeatedly (five times, 10 ml. each). Yellow colored TNP-histidine peptide in the aqueous solution was finally separated from inorganic salts, trinitrobenzene sulfonic acid and a trace amount of unreacted peptide by means of a talc-Celite column (*1*, *2*, *4*).

Identification of Histidine Peptides—An aliquot of the eluate from the talc-Celite column with a mixture of *N* hydrochloric acid and ethanol (1 : 1 v/v), containing α-mono-TNP-histidine peptide, was hydrolysed with 6 *N* hydrochloric acid at 100° to 110° for 6 hours. The resulting TNP-amino acid (in 55–65 per cent yield) from the N-terminal and free amino acid (in more than 90 per cent yield) from non-N-terminal, were semi-quantitatively identified by paper chromatography: 1.5 *M* sodium phosphate buffer (pH 5.4) and *n*-butanol saturated with 5 per cent ammonia were used as the solvents for TNP-amino acids (*4*); phenol saturated with water and a mixture of *n*-butanol, acetic acid, and water (4 : 1 : 2 v/v) were the solvent systems for free amino acids.

In order to examine the C-terminal, an aliquot of the above-mentioned eluate, after the concentration to dryness, was hydrazinolyzed and the resulting free amino acid was analyzed according to Akabori's method (*9*).

Another aliquot of the eluate was used for a determination of a relative ratio of the diazo-reaction color intensity, against the optical density at 350 mμ due to α-mono-TNP group, O. D.$^{\mathrm{Im}}$/O. D.$^{\mathrm{TNP}}$, regarding that of TNP-histidine is 1.00.

RESULTS

A suspension of heat-denatured horse globin was hydrolyzed with *Streptmyces griseus* proteinase as shown in Fig. 1. After 2 hours, all precipitates were solubilized completly and after 4 hours, all histidine-, tyrosine-, and arginine-residues were found in the 0.22 *M* trichloroacetic acid-soluble fraction under the conditions used.

From the partial hydrolyzate after 24 hours, six kinds of diazo-reaction positive components were eluted from a column of Amberlite IRC-50 (pH 5.2) with *M*/2 sodium citrate buffer and final elution with *N* sodium hydroxide gave two additional components; thus the one was eluted when the column was neutralized up to pH 6 and the other after the complete alkalization of the column (pH 10), as shown in Fig. 2. Judging from O. D.$^{\mathrm{Im}}$ of the original enzymic hydrolyzate and of the whole chromatographic effluents, at least 95 per cent of the histidine residues seemed to be recovered, and the column, after the final elution with sodium hydroxide, was scarcely positive with diazotized sulfanilic acid.

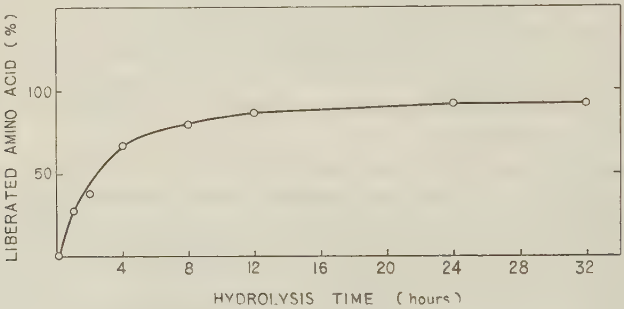

Fig. 1. Hydrolysis of horse globin with *Streptomyces griseus* proteinase. (Enzyme/protein 1/50 wt.; 37°; pH 7.5; Na· *M*/30)

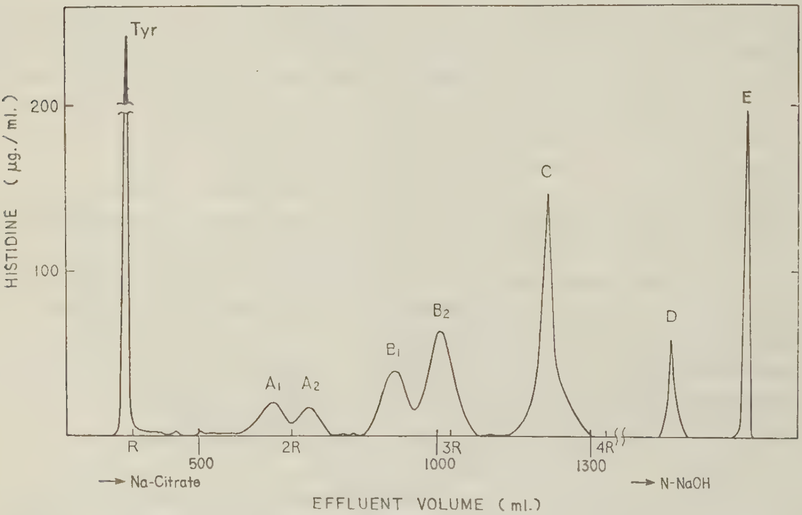

Fig. 2. Elution chromatogram of diazo-reaction positive components derived from horse globin (320 mg.) with *Streptomyces griseus* proteinase. (Amberlite IRC-50, pH 5.2, 1.8×140 cm.; *M*/2 sodium citrate at pH 5.2 then *N* NaOH)

The fraction corresponding to peak No. 1 gave only one diazo- and nitroso-naphthol-reactions positive spot on the paper chromatograms, and the R_f values were just the same as those of tyrosine. The other fractions corresponding to peaks No. 2–No. 8, on the other hand, contained no tyrosine residues. As these seven fractions were still contaminated with argine- or lysine-peptides, the former contaminants were removed by a treatment with Amberlite IRA-411 column (OH phase) (*1*, *2*), and the latters were separated as the bis-TNP-derivatives with ether from the acidified aqueous solution of TNP-histidine peptide. So purified α-mono-TNP-histidine peptides moved as a single spot on the paper chromatogram, whose R_f values are shown in Table I.

TABLE I

Yield and Some Analytical data of Histidine Peptides Derived from Horse Globin

No.1)	R_f 2)	O.D.$^{\text{Im}}$/O.D.$^{\text{TNP}}$ 3)	Yield4)	N-Terminal	non-N-terminal	C-Terminal	
1			(5.8)				Tyr
2	0.78	1.0	0.8	Ser	His		Ser-His
3	0.75	1.0	0.7	His	Gly		His-Gly
4	0.83	1.0	2.5	His	Gly, Ser	Gly	His-Ser-Gly
5	0.71	1.0	3.5	His	Ala, Ser	Ser	His-Ala-Ser
6	0.60	1.0	7.5	His	none		His
7	0.59	2.0	0.8	His	Gly, His	Gly	His-His-Gly
8	0.57	2.0	1.5	His	His		His-His
		Total.........	17.3				

1) No. of diazo-reaction positive peak on elution diagram (Fig. 2)

2) Solvent, a mixture of *n*-butanol, acetic acid and water (4 : 1 : 2 v/v) (*4*)

3) The relative ratio of color intensity with diazonized sulfanilic acid against optical desity at 350 mμ of TNP-derivative, regending that of TNP-histidine as 1.00.

4) Expressed as mole of histidine residue per 35,000 g. of globin

TNP-peptides No. 2–No. 6 gave just the same relative ratio, O. D.$^{\text{Im}}$/O. D.$^{\text{TNP}}$ as that of α-mono-TNP-histidine while the ratio of TNP-peptides No. 7 and No. 8 were twice as much, indicating that the former five were mono histidine peptides while the latter two had two histidine residues, respectively. From the analytical data on the amino acid constituents, N- and C-terminals and the above-mentioned ratio, the structure of these seven diazo-reaction positive components were elucidated. These results are summarized in Table I, together with the yield of these components calculated from O. D.$^{\text{Im}}$.

DISCUSSION

As shown in Table I, the relative ratios, O. D.$^{\text{Im}}$/O. D.$^{\text{TNP}}$ of various TNP-mono-histidine peptides isolated from horse globin, were just the same as that of α-mono-TNP-histidine, while the molar extinction coefficient of α-mono-TNP-peptide, O. D.$^{\text{TNP}}$, was slightly less than that of α-mono-TNP-amino acid (*4*, *6*). So the yield of each histidine peptide, calculated with an assumption that O. D.$^{\text{Im}}$ of histidine peptide was just the same as that of equivalent free histidine may be slightly lower than the actual value. A small discrepancy between the total histidine residues assayed from O. D.$^{\mathrm{Im}}$ of enzymic hydrolyzate (17.3 moles per 35,000 g.) and of complete hydrolyzate (19 moles per 85,000 g.) might be due to the above-mentioned reason.

From the results shown in Table I, the presence of 4 moles of His-Ala-Ser, 3 moles, of His-Ser-Gly, one mole of Ser-His and His-Gly per 35,000 g. of horse globin, was concluded. Besides these nine histidine residues, there seemed to be present at least two kinds of His-His linkages, as the presence of Ala-His-His-Arg had been already elucidated by Satake *et al*. (*10*) and as appreciable amounts of His-His (0.7 moles) and some amount of His-His-Gly (0.4 moles) were isolated in this experiment. His-Gly mentioned already, might be derived by a slow hydrolysis of His-His linkage of the latter tripeptide, though there would be another interpretations.

Recently Keil and Sôrm (*11*) obtained Ala-His, Leu-His, and (Val, His) together with His-Ala, Ser-His-Lys from the partial hydrolyzate of horse hemoglobin with concentrated hydrochloric acid. The comparison of the both data by Sôrm and by the present authors, which were not inconsistent each other, seemed to give further informations on the structure of horse hemoglobin and on the substrate specificity of *Streptomyces griseus* proteinase (*1*, *2*)

$\downarrow$ His—Ala—Ser $\downarrow$
$\downarrow$ His—Ser—Gly $\downarrow$
$\downarrow$ Ser—His $\downarrow$ Lys—
$\downarrow$ His $\overset{\frown}{\underset{\smile}{\downarrow}}$ His—Gly $\downarrow$
—Ala $\downarrow$ His $\overset{\frown}{\underset{\smile}{\downarrow}}$ His $\downarrow$ Arg $\downarrow$
—Leu $\downarrow$ His $\downarrow$
—Ala $\downarrow$ His $\downarrow$

$\downarrow$ $\overset{\frown}{\underset{\smile}{\downarrow}}$ indicates that the bond was hydrolysed with *S. griseus* proteinase.

SUMMARY

1. Histidine peptides derived from horse globin with *Streptomyces griseus* proteinase, were separated on a column of Amberlite IRC-50 (pH 5.2) and purified as the α-mono-TNP-derivatives.

2. On the acid-hydrolysis of α-mono-TNP-histidine peptides, α-mono-TNP-histidine from N-terminal and free histidine from the non-N-terminal positions, could be recovered in good yield, respectively.

3. The presences of 4 moles of His-Ala-Ser, 3 moles of His-Ser-Gly, one mole of His-His-Gly, Ala-His-His-Arg, and Ser-His per 35,000 g. of horse globin was elucidated.

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REFERENCES

(*1*) Sasakawa, S., and Satake, K., *J. Biochem.*, **45**, 867 (1958)
(*2*) Satake, K., and Luck, J. M., *Federation Proc.*, **18**, 313 (1959)

(3) Nomoto, M., and Narahasei, Y., *J. Biochem.*, **46**, 653, 839 (1959)
(4) Okuyama, T., and Satake, K., *J. Biochem.*, **47**, 454 (1960)
(5) Hirs, C. H., Stein, W. H., and Moore, S., *J. Biol. Chem.*, **200**, 493 (1953)
(6) Satake, K., Okuyama, T., Ohashi, M., and Shinoda, T., *J. Biochem.*, **47**, 654 (1960)
(7) Ceriotti, G., and Spandrio, L., *Biochem. J.*, **66**, 607 (1957)
(8) Macpherson, H. T., *Biochem. J.*, **40**, 470 (1946)
(9) Akabori, S., Ohno, K., and Narita, K., *Bull. Chem. Soc. Japan*, **25**, 214 (1952)
(10) Ozawa, H., Tamai, K., and Satake, K., *J. Biochem.*, **47**, 244 (1960)
(11) Mäsiar, P., Keil, B., and Sôrm, F., *Coll. Czechoslov. Chem. Commun.*, in press (cited in Symposium on Protein Structure ed. by Neuberger, A., John Wiley & Sons Inc., New York, p. 88 (1958))

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STUDIES ON ENZYMIC NITRITE REDUCTION

III. EFFECTS OF METAL IONS ON SOLUBLE AND PARTICULATE COMPONENTS OF NITRITE REDUCTASE

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In the previous paper (*1*), it was reported that nitrite reductase (NiR)** of a halotolerant micrococcus, strain 203, could be separated into a soluble component (SF) and a particulate component (PF). Each component by itself showed no NiR activity, but the activity appeared on recombination of the two components. It was also suggested from inhibition studies that different metals are involved in the functioning of SF and PF. The present paper describes the solubilization and purification of PF and the effect of various metal ions on the activities of PF and SF. The intracellular distribution of CO-binding pigment(s) in this micrococcus will also be briefly reported.

METHODS

Microorganism—A halotolerent micrococcus, strain 203, was cultured aerobically, harvested, and lyophilized as described by Kono *et al.* (*2*). The lyophilized cells could be stored in a vacuum desiccator at 4° for several weeks without loss of activity.

Enzyme Assay—The reaction was carried out anaerobically in Thunberg tubes. Preparations of PF and SF, phosphate buffer (pH 6.8, final concentration 0.05 *M*), and 5.0 μmoles of MBH_2 were placed in the main compartment of a Thunberg tube and 2.5 μmoles of sodium nitrite in the side arm (total volume, 2.5 ml.). The reaction was started by tipping the nitrite into the main compartment. When PMSH was used as electron donor instead of MBH_2, sodium nitrite, PF and SF were placed in the main compartment and 0.5 ml. of 0.1 *M* phosphate buffer (pH 6.8) containing 2.5 μmoles of phenazine methosulfate and 5.0 μmoles of ascorbic acid in the side arm (total volume, 2.5 ml.). Incubation was carried out at 37°. In order to estimate the activity of SF, a saturating amount of PF was added in the reaction mixture. The activity of PF was similarly determined in the presence of an excess of SF. The NiR activity was followed by measuring the disappearance of nitrite according to the method described in a previous paper (*3*).

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** Following abbreviations are used in this paper; NiR, nitrite reductase; SF, soluble component: PF, particulate component; PMSH, reduced phenazine methosulfate; MBH_2, reduced methylene blue; tris, tris (hydroxymethyl) aminomethane; HdR, hydroxylamine reductase.

Purification of SF—All purification procedures for SF and PF were performed in the cold room maintained at 4° unless otherwise stated. The purification of SF was carried out mostly as described in the previous paper (*1*). The lyophilized cells were lysed by lowering the osmotic pressure, treated with desoxyribonuclease to decrease the viscosity, and the lysed mixture was centrifuged at 20,000×*g* for 30 minutes. The bulk of SF activity was recovered in the red supernatant. The precipitate, on the other hand, contained most of the PF activity and was used for the purification of PF. SF was purified from the supernatant by acetone precipitation followed by calcium phosphate gel adsorption and elution. The purified SF preparation was then dialyzed with stirring against 250 volumes of glass-distilled water for 4 hours with two changes of water. The dialyzed preparation was used for metal activation experiments.

Purification of PF—The precipitate obtained as described above from 12 g. of lyophilized cells was suspended in 100 ml. of cold water and the suspension was centrifuged at 2,000×*g* for 10 minutes. The turbid supernatant thus obtained was again centrifuged at 20,000×*g* for 30 minutes. The precipitate was then washed several times by suspending in 100 ml. of cold distilled water and centrifuging at 20,000×*g* for 30 minutes. The washed precipitate was suspended in 100 ml. of 0.05 *M* tris buffer (pH 7.5) and exposed to sonic oscillation (10 KC) for 15 minutes at or below 8°. The sonicate was centrifuged at 20,000×*g* for 30 minutes and the bulky white precipitate was discarded. To the slightly turbid supernatant was added 100 mg. of crude snake venom obtained from *Trimersurus flavoviridis Hallowell* and the mixture was incubated for 3 hours at 16–20° with stirring. After incubation, 0.1 volume of 5 *M* sodium chloride was added to the mixture to facilitate the separation of solubilized PF from the residual particulate fractions. The mixture was then centrifuged at 105,000×*g* for 60 minutes and the red precipitate was discarded. To 100 ml. of the yellow supernatant was added 30 g. of powdered ammonium sulfate and the precipitate formed was collected by centrifugation, dissolved in a small portion of cold distilled water, and dialyzed against 250 volumes of cold glass-distilled water for 4 hours with stirring, water being changed two times. The dialyzed preparation was used for metal activating experiments.

Spectrophotometry—Spectrophotometric experiments were conducted using a Hitachi Model EPU spectrophotometer or a Cary Model 14 recording spectrophotometer. For the measurements of reduced minus oxidized difference spectra and CO-difference spectra Thunberg-type cuvettes were employed.

RESULTS

Solubilization of PF—Desoxycholate, *n*-butanol, isobutanol, *tert*-amyl alcohol, pancreatic lipase, and snake venom were tested as solubilizing agents in attempts made to obtain the PF activity in a soluble state. It was thus revealed that only the treatments with lipase and snake venom were effective in solubilizing the particulate fraction without appreciable loss of PF activity. The use of the other agents resulted in considerable loss of activity. The degree of solubilization attainable by lipase or snake venom was, however, not very high. The treatment with snake venom caused more extensive solubilization (35 per cent) than that with lipase. The snake venom treatment was, therefore, employed in most of the experiments as described under Methods.

Purification of SF and PF—The results of typical purification experiments are summarized in Table I (a) and (b). As is able to be seen from Table I (a), it was possible to purify the SF activity to an extent representing a 8.6-fold increase in specific activity (measured with MBH_2 as electron donor) with a recovery of 23 per cent. Since the preparation thus purified was still contaminated with hydoxylamine reductase, further purification was attempted by DEAE-cellulose and Dowex-2 column chromatograhy to remove the HdR activity. Only partial separation of SF from HdR was, however, achieved by these procedures. The PF activity was purified as described under Methods. As is shown in Table I (b), the specific activity as measured with MBH_2 as electron donor increased about 20-fold and the recovery of total activity was 26 per cent.

TABLE I

(*a*) *Summary of Purification of SF of Nitrite Reductase*

Fractions	Specific Activity[1]	Recovery of Total Activity (%)
Whole cell	1.7	100
20,000 × *g* supernatant	2.6	92
Acetone precipitate	7.0	46
Gel eluate	14.5	23

(*b*) *Summary of Purification of PF of Nitrite Reductase*

Fractions	Specific Activity[1]	Recovery of Total Activity (%)
Whole cell	2.3	100
Washed particle	7.7	85
Sonic extract	23.0	85
Habu venom solubilyzed	21.0	30
Ammonium sulfate precipitate	40.7	26

1) μmoles nitrite reduced/hour/mg-nitrogen

Effects of Metal Ions on SF and PF Activities—The dialyzed SF and non-dialyzed PF preparations were used in studying the effect of metal ions on the SF activity, and the non-dialyzed SF and dialyzed PF for the PF activity. The preparations used were of the highest purity attained in Table I. PMSH was employed as electron donor in these studies to exclude the participation of a third factor which is involved in the MBH_2-NiR system (*3*). The metal ion to be tested was preincubated with the enzyme preparations at 37° for 15 minutes prior to the initiation of the reaction. As will be evident from Table II, the PF activity was found to be activated by the addition of Fe^{++} and Fe^{+++} (5×10^{-5} *M*) to 180 and 250 per cent of the original activity, respectively. No activation was, however, observed by the addition of Cu^{++}, Mn^{++}, Zn^{++}, Ni^{++}, and MoO_4^{--}. The SF activity, on the other hand, was activated by Cu^{+} and Cu^{++} (5×10^{-5} *M*) (180 and 210 per cent, respectively).

Fe^{++}, Fe^{+++}, Mn^{++}, Zn^{++}, Ni^{++}, and MoO_4^{--} (5×10^{-4} *M*) showed no activation. Higher concentration of Cu^{++} (*e.g.* 10^{-4} *M*) slightly inhibited the SF activity. The SF and PF activities were inhibited 72 and 46 per cent, respectively, by 10^{-4} *M* of Cu^{++} when crude enzyme preparations were used. This may be due to the fact that the crude preparations have already been saturated with Cu^{++} and therefore the excess of Cu^{++} added to the systems exhibited its inhibitory action rather than an activating effect.

TABLE III

Activation of SF and PF of Nitrite Reductase by Metal Ions

Metal Ions	Final Concentratson (*M*)	Relative SF Activity	Relative PF Activity
None	—	100	100
Fe^{++}	5×10^{-4}	90	180
Fe^{+++}	5×10^{-4}	110	250
Cu^{+}	5×10^{-5}	180	—
Cu^{++}	5×10^{-5}	210	110
Mn^{++}	5×10^{-4}	90	100
Zn^{++}	5×10^{-4}	80	80
Ni^{++}	5×10^{-4}	65	70
MoO_4^{--}	5×10^{-4}	90	100

Intracellular Distribution of CO-Binding Pigment(s)—The NiR activity of the micrococcus is inhibited by carbon monoxide, and it was shown that the electron transfer between MBH_2 and nitrite catalyzed by the cell-free preparation requires, in additon to SF and PF, a third factor which combines with CO in the photoreversible way (*1*). The intracellular distribution of such CO-binding pigment(s) was therefore studied. Sato (*4*) was the first to discover, in this bacterium, a CO-binding pigment which has a peak at 416–417 mμ and a trough at 430 mμ in the CO-difference spectrum. Kono *et al.* (*2*) have recently succeeded in the isolation from the same organism of a soluble CO-binding pigment with an absorption maximum at 416–417 mμ in the CO-difference spectrum and reported that this pigment is autoxidizable and has a strong HdR activity.

In the present study, the lysate of the organism was fractionated by centrifugation into three fractions as described in the previous paper (*1*), namely, a particle fraction sedimented between 1,000×*g* and 20,000×*g* (P_{1-20}), another particle fraction sedimented between 20,000×*g* and 105,000×*g* (P_{20-105}), and the supernatant after the centrifugation at 105,000 *g* (S_{105}). These fractions were suspended in or mixed with phosphate buffer of pH 6.8 (final concentration 0.05 *M*) and the mixtures were anaerobically incubated at 37° for 15 minutes in the dark after being saturated with 1 atm. of CO. The spectra of these mixtures were then measured against their respective controls in which the incubation was performed in the absence of CO. It was thus revealed that a CO-binding pigment (or pigments) is present in all the three fractions, and the CO-difference spectra obtained for these fractions (Fig. 1) were almost identical in that they all show a peak at 415–416 mμ and a trough at about 433 mμ. The relative content of the CO-binding pigment(s) in each fraction was estimated from the difference in optical density between 416 mμ and 433 mμ. The ratio of CO-binding pigment content in P_{1-20} to S_{105} was thus found to be 3 : 1, while P_{20-105} contained only a trace of the pigment.

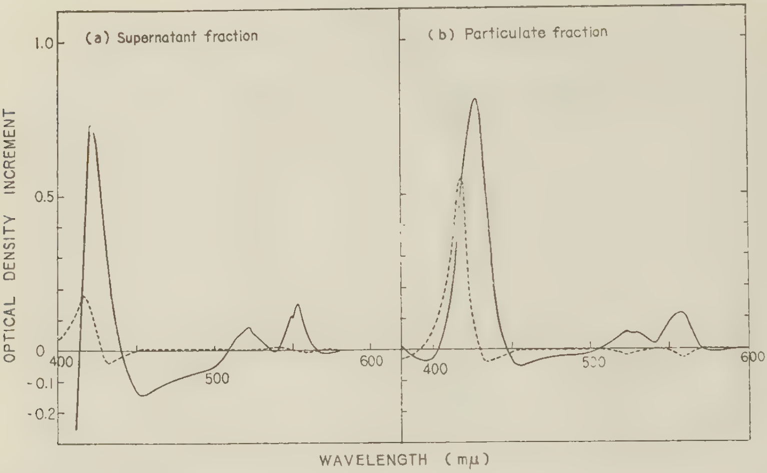

FIG. 1. CO-difference of reduced minus oxidized difference spectra of (a) supernatant fraction and (b) particulate fraction.

Cuvettes contained (a) 0.9 ml. of 105,000 × *g* supernatant or (b) 0.9 ml. of the sonicate of particle fraction (P_{1-20}), 0.15 ml. of 0.5 *M* phosphate buffer (pH 6.8) and 0.45 ml. of water. In the experimental cuvette a few crystals of sodium dithionite have been added for the measurement of the reduced minus oxidized difference spectra. CO-difference spectra were measured after the addition of a few crystals of sodium dithionite to the control cuvette and introduction of carbon monoxide (1 atm.) to the experimental cuvette followed by incubation at 37° for 15 minutes in the dark. Thunberg type cuvettes were used. — Reduced minus oxidized diference pectrum, -- CO-difference spectrum

In order to further study the CO-binding pigment(s) in S_{105}, the supernatant was fractionated by acetone precipitation and calcium phosphate gel adsorption as described for the purification of SF (*1*). The relative content of the pigment(s) in the preparations thus obtained was estimated as described above and the following figures were obtained: acetone 0–40 per cent precipitate, 18; acetone 40–50 per cent precipitate (containing most of SF and HdR), 7; acetone 50–67 per cent precipitate (containing most of cytochrome b_4), 0; fraction unadsorbed on calcium phosphate gel, 3; fraction eluted from the gel with 0.033 *M* of phosphate buffer at pH 6.8, 3.4; fraction eluted with 0.1 *M* phosphate buffer at pH 6.8 (containing most of SF and HdR), 0.6.

Whether the CO-binding pigment(s) found in these various fractions are identical or different is not yet clear.

DISCUSSION

Chung and Najjar (*5*) have reported that the reduction of nitrite in *Pseudomonas stutzeri* is activated by Cu^{++}, Fe^{++} and Fe^{+++}. They (*6*) have also found that both particulate and soluble fractions are required for maximal reduction of nitrite. However, since what was measured in their studies is the overall electron transport from reduced pyrimidine nucleotides to nitrite, it was not possible to determine if the metal ions activated the terminal nitrite reductase or the other components of the whole electron transport system. This complication was avoided in the present study by using PMSH as electron donor (PMSH is considered to react directly with the terminal NiR) and by employing partially purified enzyme preparations. It was thus established that the PF component of NiR is activated by Fe^{+++} and Fe^{++} and that the SF component by Cu^{+} and Cu^{++}.

Iwasaki and Mori (*7*) have reported that the denitrifying system of a pseudomonad could be separated into two components by ammonium sulfate fractionation. It has also been observed that one of these components could be replaced by a cytochrome isolated from other bacteria*. It seems, however, unlikely that neither SF nor PF of NiR described in this paper is of cytochrome nature. It is probable that the difference in source organisms and that in the method of enzyme assay (PMSH-nitrite system in this study and dimethyl-*p*-phenylenediamine-nitrite in theirs) may be responsible for the apparent discrepancy.

There is as yet no evidence available to elucidate the intimate mode of action of CO-binding pigments in the nitrite-reducing system.

SUMMARY

1. The particulate component (PF) of nitrite reductase of a halotolerant micrococcus, strain 203, was solubilized by the treatment with snake venom and purified by ammonium sulfate fractionation.

2. Using partially purified and thoroughly dialyzed preparations, it was found that the activity of soluble component (SF) is activated by Cu^{+} and Cu^{++} and that of particulate component (PF) is enhanced by Fe^{+} and Fe^{+++}.

3. Intracellular distribution of CO-binding pigment(s) in this organism was studied.

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* Personal communication from Dr. H. Iwasaki, Department of Biology, Faculty of Science, Nagoya University. (Details of the work were published in this Journal, Vol. **47**, 174, shortly before the preparation of proofs of the present article).

REFERENCES

(*1*) Asano, A., *J. Biochem.*, **46**, 1235 (1959)

(*2*) Kono, M., Taniguchi, S., and Egami, F., *J. Biochem.*, **44**, 615 (1957)

(*3*) Asano, A., *J. Biochem.*, **46**, 781 (1959)

(*4*) Sato, R., Unpublished observation, cited by B. Chance in "The Physical Chemistry of Enzymes." Discussions of the Farady Society, 1955, No. 20, p. 205

(*5*) Chung, C. W., Najjar, V. A., *J. Biol. Chem.*, **218**, 617 (1956)

(*6*) Najjar, V. A., and Chung, C. W., *A Symposium on Inorganic Nitrogen Metabolism*, The Johns Hopkins Press, Baltimore, p. 260 (1956)

(*7*) Iwasaki, H., and Mori, T., *J. Biochem.*, **45**, 133 (1958)

The Journal of Biochemistry, Vol. 47, No. 5, 1960

MECHANISM OF ACTION OF VIOMYCIN

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Viomycin is one of the important antituberculous agents. There is yet no report concerning the mechanism of action of viomycin. Previously, the author (*1*) reported that viomycin can precipitate both nucleic acids, deoxypentose nucleic acid (DNA) and pentose nucleic acid (RNA), especially to a great extent the latter one. This fact has suggested that viomycin may inhibit the biosynthesis of protein in bacteria. The present study was performed to examine this possibility and proved that it can occur truely.

MATERIALS AND METHODS

Test Strain—Mycobacterium avium, strain Jucho (an avirulent strain representing a S-type colonial morphology), was used throughout the study.

Medium—Sauton medium, pH 7.0, in which sodium glutamate was substituted for asparagine, was used in the study for cultivating the organism.

Preparation of Cell Suspensions—5 to 8 days old cultures growing in Sauton medium were washed three times with saline and harvested. The cells washed were homogenized by shaking with glass beads and suspended in 0.033 M phosphate buffer, pH 7.1, in 0.05 M sodium citrate solution or in phosphorus-free medium, pH 7.0, to obtain appropriate final concentrations. The phosphorus-free medium consisted of 20 ml of glycerol, 2.0 gm of sodium glutamate, 1.0 gm of sodium citrate, 0.25 gm of magnesium sulfate, 0.25 gm of potassium chloride, 0.025 gm of ferrous sulfate, and 980 ml of distilled water.

To the cell suspensions were added appropriate amounts of isotopes and viomycin, and the suspension was incubated at 37°. Viomycin sulfate manufactured by Parke & Davis Company was used throughout the study.

Fractionation of Phosphorus Compounds—After an appropriate period of incubation, the cells were collected by centrifugation and washed three times with distilled water. The cells thus obtained were fractionated by the procedure of Schneider (*2*).

Fractionation of Sulfur Compounds—The cells were collected by centrifugation and then washed four times with distilled water. The cells were extracted three times with 10 per cent trichloroacetic acid (TCA). The extract was designated as TCA-soluble fraction. The residue was dissolved in 2 per cent sodium hydroxide by heating in a water bath. The resulting fluid was designated as TCA-insoluble or protein fraction.

Isotopic Techniques—Isotopic tracer techniques were utilized to observe phosphorus and sulfur metabolisms. P^{32}-orthophosphate (manufactured by the Radiochemical Center, Amersham, England) and S^{35}-sulfate (manufactured by the Commissariat a l'Energiew Atomique, Saclay, France) were used. To determine the radioactivity of each fraction, samples of 0.2 ml. were dried on stainless planchets and counted by means of a Geiger Mueller counter (Philips, Holland). The amounts of incorporated isotopes were expressed by the term of "counts per minute" (cpm) per 1 μg. of DNA, since the amount of DNA remained unchanged throughout all the experiments.

Determination of Amounts of DNA and RNA—The amounts of DNA and RNA were determined by diphenylamine reaction (*3*) and orcinol-HCl reaction (*4*), respectively. Assays were conducted on samples of the nucleic acid fraction obtained by Schneider's procedure.

RESULTS

1. Effect of Viomycin on the Incorporation of Radioactive Phosphorus (P^{32}) into Cellular Fractions.

Incorporotion of P^{32} into the acid-soluble fraction was not significantly inhibited by the presence of viomycin. On the other hand, incorporations of P^{32} into the nucleic acid fraction and into the protein fraction were inhibited by the presence of viomycin (Table I).

TABLE I

Distribution in Cellular Fractions of Radioactivity from Incorporated P^{32} in the Presence and the Absence of Viomycin[1]

Fraction	Radioactivity as cpm per 1 μg. of DNA			
	Incubation Time: 1 Hour		Incubation Time: 3 Hour	
	Control (No Drug)	Viomycin (5 μg./ml.)	Control (No Drug)	Viomycin (5 μg./ml.)
Acid-Soluble	5.08	4.34	2.90	4.52
Phosphatide	—	—	—	—
Nucleic Acid	15.6	11.4	14.5	10.2
Protein	3.13	0.43	1.60	0.58

1) Reaction system (4.0 ml. in total):
Cells (suspended in the phosphorus-free medium) .... 40 mg./ml.
P^{32}-Orthophosphate .... ca. 1 μc./ml.
Viomycin sulfate ....0 or 5 μg./ml.

2. Effect of Viomycin on the Ratio of RNA : DNA.

The amounts of DNA and RNA in the nucleic acid fraction were determined by the chemical assays. The RNA : DNA ratio sometimes increased by the presence of viomycin. However, this finding was incostant. The ratio sometimes remained unchanged.

3. Effect of Viomycin on Incorporation of Radioactive Sulfur (S^{35}) into Cellular Fractions.

Incorporations of S^{35} into the TCA-soluble fraction and into the protein fraction were significantly inhibited by the presence of viomycin (Table II). The inhibition occurred after 1 hour of incubation. Furthermore, it was noted that ratio of specific radioactivity, expressed as cpm per 1 μg. of DNA, of the protein fraction against that of the TCA-soluble fraction was lower in the presence of viomycin than in the absence of it (Table II). Rates of inhibition were more significant in the citrate solution than in the phosphate buffer solution. The fact agreed with the previous observation indicating that the antimycobacterial action of viomycin was reduced with increasing concentrations of phosphate (*5*).

TABLE II

Distribution in Cellular Fractions of Radioactivity from Incorporated S^{35} in the Presence and the Absence of Viomycin

Reaction System	Fraction	Radioactivity as cpm per 1 μg. of DNA			
		Incubation Time: 1 Hour		Incubation Time: 3 Hour	
		Control (No Drug)	Viomycin (5 μg./ml.)	Control (No Drug)	Viomycin (5 μg./ml.)
I[1)]	Acid-Soluble	145.	117.	94.5	74.6
	Protein	136.	52.3	131.	85.2
II[2)]	Acid-Soluble	26.2	12.8	39.0	14.1
	Protein	19.4	6.0	40.3	8.1

1) Reaction system I (4.0 ml. in total)

Cells (suspended in 0.033 *M* phosphate buffer, pH 7.1)	10.4 mg./ml.
S^{35}-Sulfate	ca. 13 μc./ml.
Viomycin sulfate	0 or 5 μg./ml.

2) Reaction system II (4.0 ml. in total):

Cells (suspended in 0.05 *M* sodium citrate solution)	17.5 mg./ml.
S^{35}-Sulfate	ca. 13 μc./ml.
Viomycin sulfate	0 or 5 μg./ml.

DISCUSSION

The incorporation of P^{32} into the nucleic acid fraction was to some extent inhibited by the presence of viomycin. The incorporation of P^{32} and that of S^{35} into the protein fraction were significantly inhibited by viomycin. In view of the results, it is conceivable that viomycin inhibits the biosynthesis of protein. Previously, the author (*1*) observed that viomycin could precipitate RNA. Considering this fact, it is suggested that the inhibition of protein synthesis by viomycin may be derived from damaged functioning of RNA. In addition, it is considered that phosphorus turnover in the nucleic acids is suppressed by viomycin, for it was shown that incorporation of P^{32} into the nucleic acid fraction was also to some extent inhibited by the presence of viomycin.

SUMMARY

The mechanism of action of viomycin was studied by utilizing isotopic techniques and *Mycobacterium avium* (Jucho strain).

The incorporation of P^{32} into the nucleic acid fraction and into the protein fraction were inhibited by viomycin, and the incorporation of S^{35} into

the protein fraction was inhibited by viomycin. It is suggested that viomycin inhibits the biosynthesis of protein and this inhibition may be resulted from damaged functioning of RNA, considering the author's previous observation that viomycin precipiatates RNA.

REFERENCES

(*1*) Tsukamura, M., and Tsukamura, S., *J. Biochem.*, **46**, 1193 (1959)
(*2*) Schneider, W. C., *J. Biol. Chem.*, **161**, 293 (1945)
(*3*) Dische, Z., *Mikrochemie*, **8**, 4 (1930)
(*4*) Kerr, S. E., and Seraidarian, K., *J. Biol. Chem.*, **59**, 211 (1945)
(*5*) Tsukamura, M., *Medicine and Biology* (Japan), **51**, 140 (1959)

The Journal of Biochemistry, Vol. 47, No. 5, 1960

THE SYNTHESIS OF FATTY ACIDS IN THE CELL-FREE EXTRACT OF MYCOBACTERIUM AVIUM

It has long been recognized that tubercle bacilli contained a high concentration of lipids as cellular constituents. Extensive works of Anderson and his colleagues (*1*) and the more recent studies of other investigators (*2*) revealed the chemical nature of lipids in tubercle bacilli and other mycobacteria. Furthermore, these lipids have been shown to have characteristic biological or pathogenic effects in animal bodies (*3–5*). However, the mode of biosynthesis of lipids in these organisms still remains to be established. Recently, we have succeeded in obtaining the cell-free extract incorporating labeled acetate or malonate into long-chain fatty acids from *Mycobacterium avium* (*6*).

Mycobacterium avium, strain Takeo, was grown and cell-free extract was prepared, as described before (*7*), except that 0.01 *M* tris (hydroxymethyl) aminomethane buffer (pH 7.8) was used instead of distilled water. Two ml. of this extract was then incubated with acetate-1-C^{14} (2.8×10^5 c.p.m.), 5 μ moles of ATP, and 80 μ moles of phosphate buffer (pH 7.5) in a total volume of 3.0 ml. for two hours at 30°. The reaction was stopped by adding 3.0 ml. of 10 per cent alcoholic NaOH, and the mixture was refluxed in a boiling water-bath for 40 minutes, then adjusted to pH 2 with 6 *N* HCl. The fatty acids in the mixture were extracted three times with 5 volumes of petroleum ether, and the extracts were combined, evaporated, and then counted for radioactivity. As shown in Table I (Experiment 1), labeled acetate was incorporated remarkably into fatty acids by the cell-free extract. Without ATP, the extent of the incorporation was greatly diminished. Since the extract is the supernatant obtained by centrifugation at $144{,}000\times g$, the enzyme system responsible for this incorporation seems to be present in the soluble state. Only a small extent of acetate incorporation was observed in the presence of the particle fraction described previously (*8*) as shown in Table I (Experiment 2). Moreover, Table I (Experiment 3) indicates that the enzyme system was precipitated between 0.3 and 0.6 saturation of ammonium sulfate from the soluble fraction. In order to identify the products synthesized from labeled acetate, the mixture of fatty acids extracted with petroleum ether was converted to their *p*-bromophenacyl ester 2,4-dinitrophenylhydrazones, which were submitted to reversed-phase paper chromatography described by Inoue *et al.* (*9*). The vertical strips including the yellow zones of fatty acid derivatives from C_8 to C_{18} were cut from the paper and extracted with benzene. When the radioactivity of the benzene extract was determined, labeled acetate was seen to be incorporated predominantly into palmitic acid, and besides, to some extent, into other long-chain fatty acids, as shown in Fig. 1. The fatty acids synthesized from labeled acetate were also fractionated using reversed-phase partition chromatography

TABLE I

Incorporation of Acetate-1-C^{14} into Fatty Acids

In Experiment 1, the experimental conditions are described in the Text. In Experiment 2, after grinding cells with sea-sand, the mixture was centrifuged at 15,000×*g* for 60 minutes, and the supernatant obtained was then resolved into both soluble and particle fractions by centrifugation at 144,000×*g* for 60 minutes. Other conditions are the same as in Experiment 1. In Experiment 3, the precipitate between 0.3 and 0.6 saturation of ammonium sulfate from the extract was used. Complete system contains 0.1 ml. 0.5 *M* $KHCO_3$. Other conditions are the same as in Experiment 1.

Experiment	Conditions	Radioactivity incorporated into fatty acids
1	Complete system	6,399 cpm
	ATP omitted	1,877
2	Soluble fraction	35,829
	Particle fraction	351
	Combined	22,720
3	Complete system	5,946
	$KHCO_3$ omitted	1,477
	ATP omitted	35

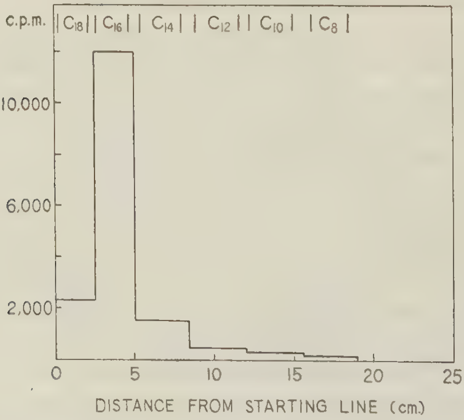

FIG. 1. Paper chromatography of the *p*-bromophenacyl ester 2,4-dinitrophenylhydrazones of the fatty acids synthesized from acetate-1-C^{14} by the cell-free extract of *M. avium*. Aliquots containing 15,000 c.p.m. were put on Toyo No. 2 filter paper impregnated with decaline, developed with 90 per cent aqueous ethanol-acetic acid-decaline (10 : 2 : 1), and the radioactivity was measured as described in the Text.

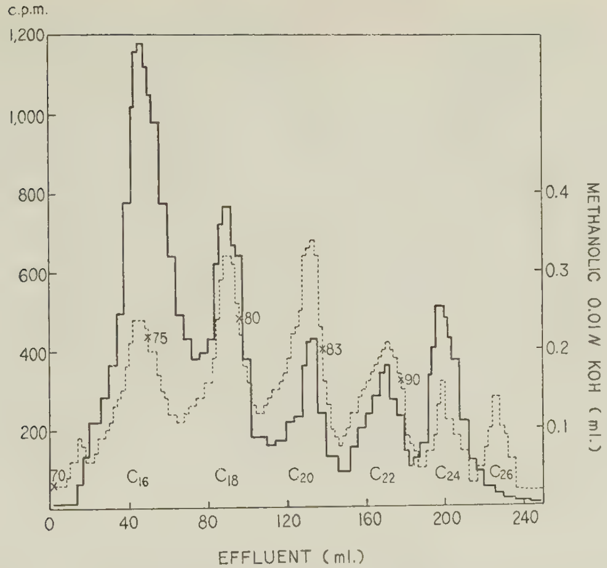

FIG. 2. Chromatogram of the fatty acids synthesized from acetate-1-C^{14} with a known amount of even-numbered fatty acids as carriers. The system is liquid paraffin on non-wetting kieselguhr, developed with acetone containing varying amounts of water. Solid line: C^{14} activity curve. Dotted line: titration curve of added even-numbered fatty acids from C_{16} to C_{26}. The marks of "X" represent points of change to higher concentrations of aqueous-acetone developing solvent containing the volume per cent acetone indicated by the number.

TABLE II

Effects of Carbon Dioxide Absorber and Malonate on the Incorporation of Acetate-1-C^{14} into Fatty Acids in the Cell-free Extract

The components of the reaction mixture were the same as in the Text except that 0.1 ml. of 0.1 *M* malonate was added as indicated below. The reaction was carried out in the Warburg cup. In Experiment 1, 0.3 ml. of 20 per cent KOH was added in the center well to absorb CO_2. In Experiment 2, KOH was absent. The fatty acids were resolved by the reversed-phase chromatography as described in Fig. 2.

Experiment	Addition	Radioactivity incorporated into					
		Palmitic acid C_{16}	Stearic acid C_{18}	Arachidic acid C_{20}	Behenic acid C_{22}	Lignoceric acid C_{24}	Total fatty acid, sum
		cpm	cpm	cpm	cpm	cpm	cpm
1	None	7,627	2,887	1,144	1,089	1,862	14,609
	Malonate	3,488	1,497	1,332	2,110	8,807	17,234
2	None	16,927	8,344	3,567	3,717	4,627	37,182
	Malonate	6.154	3,822	2,337	3,826	16,663	32,807

devised by Silk and Hahn (*10*). Again, it was indicated, from the result of Fig. 2, that palmitic acid was the major product from acetate.

Previously, we found that the extract of *M. avium* catalyzed carbon dioxide fixation into malonate (*7*). In addition, Wakil (*11*) and Brady (*12*) postulated that malonyl coenzyme A or its derivative would participate as essential intermediate in fatty acid synthesis in animal tissues. Therefore, the effect of carbon dioxide or malonate in the fatty acid synthesizing system of *M. avium* was examined. Table II indicates clearly that the amounts of the incorporation into fatty acids were greater in a cup without alkali than with alkali in a center-well of the Warburg manometer. Moreover, the requirement of bicarbonate in this system was revealed by ammonium sulfate precipitation of the extract, as in Table I (Experiment 3). Further, it is of great interest that in the presence of malonate most radioactivity appeared in the fraction of the fatty acids higher than palmitic acid, possibly lignoceric acid, as summarized in Table II. A similar result was obtained, when malonate-1-C^{14} was used instead of acetate-1-C^{14}.

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REFERENCES

(*1*) Anderson, R. J., *Physiol. Rev.*, **12**, 166 (1932), *Chem. Rev.*, **29**, 225 (1941)
(*2*) Asselineau, J., and Lederer, E., Ciba Foundation Symposium on Experimental Tuberculosis, p. 14 (1955)
(*3*) Noll, H., and Bloch, H., *J. Biol. Chem.*, **214**, 251 (1955)
(*4*) Yamamura, Y., *Adv. Tuberc. Res.*, **9**, 13 (1958). Yamamura, Y., Yasaka, S., Endo, K., Iwakura, H., Nakamura, S., and Ogawa, Y., *Proc. Japan Acad.*, **31,** 36 (1955)
(*5*) Weiss, D. W., and Dubos, R. J., *J. Exper. Med.*, **163**, 73 (1956)
(*6*) Kusunose, M., Kusunose, E., and Kowa, Y., The abstract of the 3 rd Meeting of Biochemists for Tuberculosis Research, November 4, p. 9 (1959)
(*7*) Kusunose, M., Kusunose, E., Kowa, Y., and Yamamura, Y., *J. Biochem.*, **46,** 525 (1959)
(*8*) Kusunose, M., Nagai, S., Kusunose, E., and Yamamura, Y., *J. Bacteriol.*, **72,** 754 (1956)
(*9*) Inoue, Y., Hirayama, O., and Noda, M., *Bull. Agri. Chem. Soc., Japan*, **20**, 200 (1956)
(*10*) Silk, M. H., and Hahn, H. H., *Biochem. J.*, **46**, 532 (1954)
(*11*) Wakil, S. J., *J. Am. Chem. Soc.*, **80**, 6465 (1958) Wakil, S. J., and Ganguly, J., *J. Am. Chem. Soc.*, **81**, 2597 (1959)
(*12*) Brady, R. O., *Proc. Nat. Acad. Sci.*, **44**, 993 (1958) Formica, J. V., and Brady, R. O., *J. Am. Chem. Soc.*, **81**, 752 (1959)

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SHAPE TRANSFORMATION OF ERYTHROCYTE GHOSTS ON ADDITION OF ADENOSINE TRIPHOSPHATE TO THE MEDIUM

In a previous paper, the authors reported that the human erythrocytes, which had become spherical at the end of eight week storage in acid-citrate-dextrose solution, were transformed to a shallow bowl form on incubation with added adenine and inosine. This phenomenon was accompanied by a marked rise in adenosine triphosphate (ATP) content of the cells. While intact cells of the aged blood remained spherical after the addition of ATP, ghosts obtained by moderately hypotonic hemolysis changed into a certain characteristic shape by the addition of ATP.

Immediately after 7 ml. of cold distilled water was added slowly to 3 ml. of acid-citrate-dextrose (ACD) blood stored for over 8 weeks, the hemolysate was centrifuged at 10,000 r.p.m. at room temperature for 3 minutes. After washing twice with cold physiological saline solution, the precipitated ghosts were resuspended in a physiological saline solution containing one tenth volume of tris(hydroxymethyl)-aminomethane buffer at pH 8.0. The behaviour of the ghosts was observed in hanging drop preparations. The

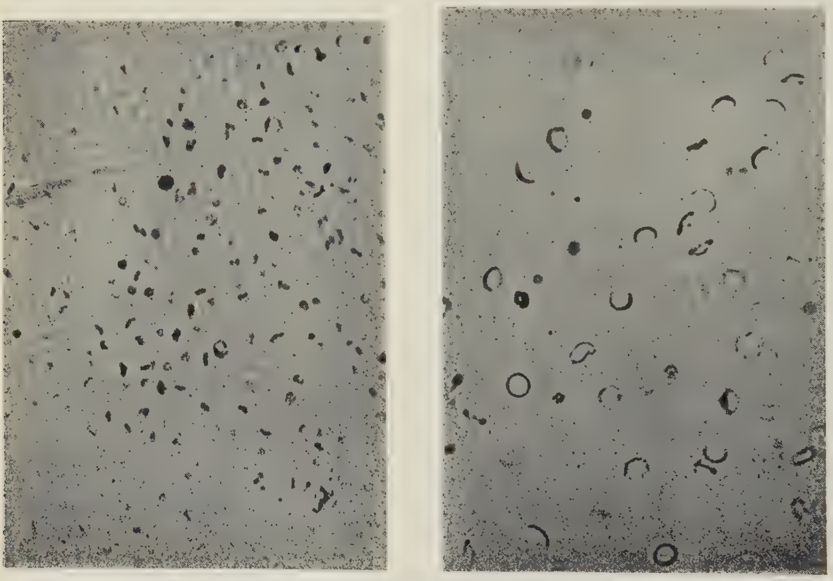

FIG. 1. Microphotographs of ghosts obtained from ACD blood stored for 8 weeks. Left: Without ATP; Right: After the addition of ATP to 0.04 *M*/l. Magnification is the same.

ghosts obtained from so-long stored erythrocytes appeared to be small shrunk bodies and the shape transformation of them took place within a few minutes after the addition of 0.003–0.04 M or more of adenosine triphosphate, as illustrated in Fig. 1. On diluting the medium with physiological saline or adding ethylenediamine tetraacetate (EDTA) to a concentration of 0.004 M the shape of the ghosts returned to nearly the original. Both sodium and potassium salts of adenosine triphosphate were equally effective, as were the same amount of adenosine diphosphate and the higher concentration of inorganic orthophosphate. On the other hand, the addition of adenosine monophosphate, adenine, or inorganic pyrophosphate did not induce the shape transformation. The ghosts responded to ATP in 1.15 per cent potassium hydrochloride solution essentially to the same extent as in 0.9 per cent sodium hydrochloride solution. However, the pH effect was rather marked. While the phenomenon took place at pH 7.8–8.4, the apparent swelling of the ghosts were sometimes observed at pH of about 7 after the addition of EDTA and the apparent shrinking after the addition of ATP. 10^{-4} M of monoiodoacetate and 10^{-5} M of *p*-chloromercuribenzoate inhibited markedly the action of adenosine triphosphate. Similar shape transformation was also observed in glycerol-treated erythrocytes, which were prepared from the blood stored for more than 8 weeks. An attempt to extract a contractile protein from the material is now in progress.

REFERENCES

(*1*) Nakao, M., Nakao, T., Tatibana, M., Yoshikawa, H., and Abe, T., *Biochim. et Biophys. Acta*, **32**, 564 (1959)

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IMMUNOCHEMICAL STUDY OF CATALASE

In a crude preparation the quantitative determination of catalase on the basis of its activity seems to be easily disturbed by the simultaneous existence of many factors which influence its enzymic reaction. In order to exclude such an interference and to obtain a more precise knowledge about the actual amount of this enzyme protein, we have investigated its estimation by means of the quantitative precipitin reaction which has by Deutsch *et al.* (*1*) been recognized to be the available methods for the microdetermination of catalase. As to each and the same preparation the amount of catalase estimated immunochemically (PU) was compared with that calculated in terms of its enzyme activity (AU), which gave us many interesting informations. This communication deals with the results of such a study, details of which will be published later.

Catalase of human erythrocytes was highly purified in its crystalline form by the method of Herbert and Pinset (*2*). The preparation was pure electrophoretically and ultracentrifugically. Its k_1 value was 1.8×10^8 which was considered to be pure enough to be used as an antigen for the immunochemical determination.

Rabbits were innoculated intramuscularly with this purified catalase well suspended in Freund's adjuvant solution. Only a single injection administrting 10 mg. of catalase to a rabbit was proved enough to produce a fairly potent antiserum in a period of 4 to 6 weeks. The antisera pooled were inactivated at 56° for 30 minutes, followed by lyophilization, and the resulting dried sera were dissolved in distilled water at a certain concentration just prior to use. Employing an excess of this anti-catalase solution, the amount of catalase in any preparation was determined by the quantitative precipitin reaction. Details of the procedures were the same as those reported by one of us for the determination of horse serum γ-globulin (*3*).

A calibration curve for precipitin reaction was established by the use of the known and increasing amount of the crystalline catalase. The evaluation of the catalase activity was made according to the method of Bonnichsen *et al.* (*4*).

The active site on a molecule of catalase which participates in the combination with antibody seems different from the prosthetic group responsible for its function. It was supported by the fact that cyanide or cysteine, even in a definite concentration enough to suppress the activity of catalase completely, showed scarecely an inhibition against the precipitin reaction.

Estimation of Catalase in Human Erythrocytes—The hemolysate of human erythrocytes after centrifugation at $14{,}000\times g$ for 15 minutes to eliminate a non-specific precipitation, was subjected to the immunochemical determination of the quantity of catalase as well as to the evaluation of its activity. As demonstrated in Fig. 1, PU and AU exhibited a good correspondence with each other. In a case of congenital deficiency of catalase (*5*, *6*) there occurred no precipitation, confirming the results of Otani (*7*).

Estimation of Catalase in Rat Erythrocytes—The above described antiserum was found to make a remarkable cross reaction with rat or bovine catalase, but not with rabbit one. The examination on rat erythrocytes revealed that PU of rat erythrocytes was 70 per cent of PU of human erythrocytes at a same activity as shown in Fig. 1.

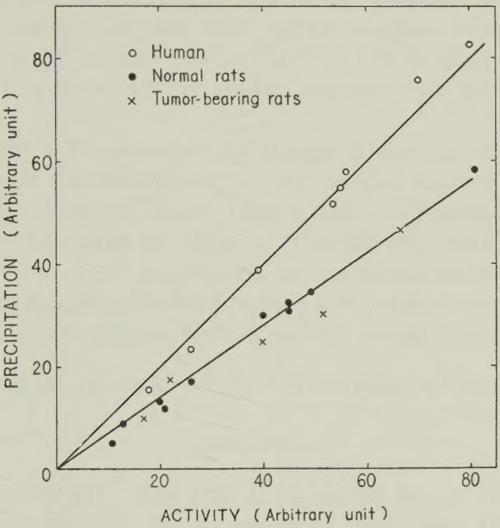

Fig. 1. The relationship between PU and AU in human and rat erythrocytes. Values are expressed in an arbitrary unit.

A supernatant solution, after centrifugation of the specific precipitate, revealed no detectable activity of catalase, and when the precipitate was suspended in 3 *M* NaCl, a reasonable activity was observed indicating the dissociation of antigen-antibody complex.

Catalase in Rat Liver—The investigation on catalase in rat liver preparations gave the following evidences indicating a discrepancy between PU and AU.

a) The ratio of PU to AU in liver homogenates was different from individual to individual, ranging rather widely from 1 : 1 to over than 3 : 1.

b) Higher ratios were observed in liver homogenates of acsites hepatoma bearing rat so far examined.

c) In extracts of ascites tumor cells a measureable precipitation took place by the antiserum, while there was no detectable activity of catalase.

d) Considering a fact that the activity of catalase in liver homogenates often changed remarkably by its standing, PU and AU before and after standing were determined. In one prepapration the ratio of PU to AU made an alteration from 1.03 to 2.08 after standing and in another case from 3.42 to 1.30, giving a reverse change.

The treatment of liver homogenates with sodium deoxycholate in tris-hydroxyaminomethane buffer (pH 8.0) as a final concentration of 0.5 per cent caused a release of catalase involved in the particulate fractions which was clearly demonstrated by an increase in both precipitin reaction and activity. This amount of catalase, designated as the total catalase (*cf.* (*8*)), was accounted two to three times greater than the soluble catalase which was detected in non-treated homogenates. Having been incubated at 37° for varying period of time, rat liver slices were homogenized and the determinations of PU and AU were made as to the total and the soluble catalase. It was realized that the ratio of PU to AU in the total catalase increased steadily with time and on the contrary, that in the soluble catalase gradually decreased.

These experimental reselts suggest the existence of a protein which shows none or little of enzyme activity but is precipitable by the antiserum against catalase. In one possibility, this protein would be expected to be an inactivated catalase, and alternatively, it might be supposed as a closely related protein to the catalase including an apocatalase, any of compounds on the way of catalase metabolism, or a certain kind of hemoproteins. The identification and the characterization of this protein is now under investigation.

We wish to express our thanks to Prof. N. Shimazono for his kind suggestion.

REFERENCES

(*1*) Deutsch, H. F., and Seabra, A., *J. Biol. Chem.*, **214**, 455 (1955)
(*2*) Herbert, H., and Pinset, J., *Biochem. J.*, **43**, 203 (1948)
(*3*) Higashi, T., *J. Japan. Biochem. Soc.*, **30**, 472 (1958)
(*4*) Bonnichsen, R. K., Chance, B., and Theorell, H., *Acta Chem. Scand.*, **1**, 685 (1947)
(*5*) Takahara, S., *Lancet*, Dec. 6, 1101 (1952)
(*6*) Kajiro, K., Kikuchi, G., Nakamura, H., and Yoshiya, M., *Chem. Ber.*, **85**, 886 (1952)
(*7*) Otani, T., *J. Japan. Biochem. Soc.*, **32**, 216 (1960)
(*8*) Adams, D. H., and Burgess, E. A., *Biochem. J.*, **71**, 340 (1958)

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